INTERLEUKIN-1, A PIVOTAL MEDIATOR IN MURINE ARTHRITIS

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(met een samenvatting in het Nederlands)

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INTERLEUKIN-1, A PIVOTAL MEDIATOR IN MURINE ARTHRITIS

INTERLEUKINE-1, EEN CENTRALE MEDIATOR IN GEWRICHTSONTSTEKINGEN BIJ DE MUIS

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CONTENTS

AA	adjuvant arthritis
AIA	antigen induced arthritis
AIL-1, AIL1	anti-IL-1
ATNF	anti-TNF
CFA	complete Freund's adjuvant
ConA	concanavalin-A
cpm	counts per minute
CIA	type II collagen induced arthritis
DTH	delayed type hypersensitivity
GAG	glycosaminoglycan
IC	immune complex
ICA	immune complex arthritis
ICE	IL-1 converting enzym
IFA	incomplete Freund's adjuvant
IFN	interferon
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL-1	interleukin-1
IL-1RI, IL-1RII	IL-1 receptor type I, II
IL-2	interleukin-2
IL-6	interleukin-6
IL-1ra, IRAP	interleukin-1 receptor antagonist
mBSA	methylated bovine serum albumin
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
OA	osteoarthritis
OVA	ovalbumin
PBS	phosphate buffered saline
PG	proteoglycan
PG-APS	peptidoglycan polysaccharide
PGE_2	prostaglandin-E_2
PHA	phytohaemaglutinin
PMN	polymorphonuclear cell
RA	rheumtoid arthritis
SCW	streptococcal cell wall
SI	stimulation index
^{99m}Tc	^{99m}Technetium pertechnetate
TGF	transforming growth factor
Th1, Th2	T-helper lymphocyte type 1, 2.
TNF	tumor necrosis factor
ZIA	zymosan induced arthritis

CHAPTER 1

ARTHRITOGENIC CHARACTERISTICS OF INTERLEUKIN-1: INTRODUCTION AND OUTLINE OF INVESTIGATION

1.1 Interleukin-1, the scope of this study.

Rheumatoid arthritis (RA) is a systemic illness with polyarticular manifestations of chronic inflammation, frequently located in the small joints of hand and feet. Among the Dutch population about 1-2% has this disease and women are more frequently (2:1) affected than men [1]. The disease is further characterized by an intermittent course of the inflammation in 70% of the patients [2].

The pathological features of RA are cartilage loss (joint space narrowing), erosions of the cortex of bone, sometimes followed by new bone formation (osteophytes) in an attempt at repair. Arthropathological changes are also observed in the joint capsule, e.g. synovial hyperplasia, hyperthrophied synovial lining layer, angiogenesis in the synovial membrane and outgrowth of the synovial membrane over the cartilage surface (pannus).

In contrast to findings in osteoarthrosis, the arthropathological changes coincide with an active synovitis. It was shown that synovectomy temporarily arrested the progressive destruction of bone and cartilage in RA [3]. An important finding since this indicates that the inflamed synovium is likely to participate in this process in the arthritic joint.

Co-cultures of cartilage and synovial tissue resulted in severe chondrocyte mediated breakdown of cartilage proteoglycans. The responsible factor was isolated and termed catabolin [4]. Cartilage destruction was also shown with a factor derived from stimulated human monocytes [5-7]. Catabolin and the mononuclear cell factor were identified as the same cytokine (extracellular soluble mediators) and received the name, interleukin-1 (IL-1).

Interleukin-1 is produced by cells of almost all lineages, binds specifically to receptors on target cells and triggers them to respond. A summary of the properties of IL-1 will be reviewed in chapter 1.2. High amounts of IL-1 are present in rheumatoid synovial fluid and the levels correlated with the disease activity in these patients (chapter 1.3). In the rheumatoid joint, tumor necrosis factor (TNF)-α seems to drive the IL-1 production [8]. Evidence for IL-1 production in animal models of arthritis are summarized in chapter 1.4.

The objective of our study was to elucidate the role of IL-1 in murine models of arthritis (chapter 1.5).

1.2 Introduction of IL-1 and level of modulation.[1]

Two forms of IL-1 exist and both are non-glycosylated proteins of 17.5 KDa with different isoelectric points, IL-1α, pI 5; IL-1ß, pI 7. Both IL-1 subtypes are encoded by two tightly linked genes which are located at chromosome 2 of man and mouse [21]. The homology of IL-1α with IL-1ß is 40-45% at the nucleic acid level and 22-26% at the amino acid level [22]. Both IL-1 forms share the same receptors and biological activities, although the immune response may be differently regulated by them [23]. Furthermore, IL-1 is well conserved between the species, a 71% homology at the amino acid level for IL-1α and 76% for IL-1ß between mouse and human, and no cross species differences are found sofar.

Most IL-1 producing cells also express a 24 KDa glycosylated IL-1 receptor antagonist protein (IL-1ra), also called IRAP or IL-1gamma, even in response to IL-1 [24,25]. For example, chondrocytes are able to produce both IL-1 and IL-1ra, suggesting an autocrine IL-1 production and regulation [26]. IL-1ra is also encoded at chromosome 2 of mice and man and has 20-30% and 19% amino acid homology to IL-1ß and IL-1α, respectively. IL-1ra competes with the IL-1 isoforms to the same receptors, however, binding does not result in signal transduction [27,28]. Recently it was demonstrated e.g in macrophages and fibroblast that an intra-cellular IL-1ra form is synthesized which theoretically can interfere with IL-1 induced responses.

CELLULAR PROCESSING OF IL-1

The IL-1 genes are transcribed into mRNA, translated into intracellular 31 KDa precursor peptides, and processed via a 22 KDa transient intracellular protein into a 17 KDa extracellular mature protein. The phosphorylated proform of IL-1α but not IL-1β is biologically active whereas both mature forms are biologically active [29].

The expression of the three IL-1 moieties (α, ß, IL-1ra) is probably regulated in a complex and by different pathways and the exact mechanism is not known up till now [30]. A central role is played by protein kinase-C (PKC) in the gene transcription of

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[1] Most of this chapter is a compilation of previous described reviews [9-20].

IL-1ß but not IL-1α, and not in the translation and secretion of both IL-1 proteins [31-34]. Both IL-1 forms lack a N-terminal signal peptide sequence that would normally indicate a natural cleavage site characteristic for secretory peptides and do not have a hydrophobic transmembrane sequence as seen in membrane proteins. In the latest hypothesis[2] a pair of two IL-1 converting enzyme (ICE) molecules are embodied in the cell membrane, forming a gap through which pro-IL-1ß passes the cell membrane under enzymatic cleavage by ICE into its mature and biologically active 17.5 KDa form. Sofar IL-1α specific converting enzyme has not been identified. Evidence is presented that pro-IL-1α is attached to the outside of the cell membrane by a lectin-like binding [35,36] but others presented data arguing against this [37]. The protease responsible for the extracellular cleavage of the proform into the mature form of IL-1α is not identified although a calpain-like protease has this capacity [38].

THE TWO IL-1 MEMBRANE RECEPTORS

The two IL-1 subtypes are recognized by the same IL-1 receptors; type I receptor (IL-1RI), a 80 KDa glycoprotein; type II receptor (IL-1RII), a 68 KDa glycoprotein [39]. The receptor numbers may differ per celltype ranging from 50 upto several thousands per cell. Both receptors possess an intracellular domain, although the IL-1RII has only a short cytoplasmic segment. The transmembrane signal transduction and the intra-cellular second messenger pathway are not unravelled. Most cells (macrophage, PMN) express both IL-1 receptors although their relative numbers may differ. The TH1 and TH2 lymphocyte populations not only have specific cytokine profiles but the relative numbers of the two IL-1 receptors are differently expressed [40-42]. Evidence is emerging that only the IL-1RI mediates signal transduction possibly by increasing cAMP and expression of the oncogenes C-myb and C-myc. However, IL-1 induced phosphorylation of protein-kinase C and oncogene expression of C-fos mediated by the IL-1 type II receptor was reported. For instance, B-cells exclusively express IL-1RII and still are responsive to IL-1 [43]. Chondrocytes express probably only IL-1RI [44-46].

It is estimated that about 1-2% of IL-1RI occupation with IL-1 is sufficient to result in a response. IL-1ra binds to the type I

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[2] Plenary lecture of M Gowen, ORS februari 21-24, 1994.

IL-1 receptor with almost the same affinity as IL-1α and IL-1β. The IL-1RII demonstrate the following affinity hierarchy; IL-1ß > IL-1α = IL-1ra. As an example, IL-1ß has a 15 times higher affinity compared to IL-1α in B-cells and in immature B-cells it is even 1000 times higher. Recently, a naturally occuring dimerization of IL-1 type II receptors is found. This complex demonstrate a higher affinity for IL-1α than the monomeric form, but affinity for IL-1β is unchanged [47]. IL-1ra binds the membrane form of IL-1RI 100 times better than the IL-1RII and binds to the soluble IL-1RI but not to the soluble IL-1RII. It is shown that the PMN cells can release their IL-1RII and that the soluble IL-1 type II receptors are a decoy for IL-1β but not for IL-1ra or IL-1α [48-50].

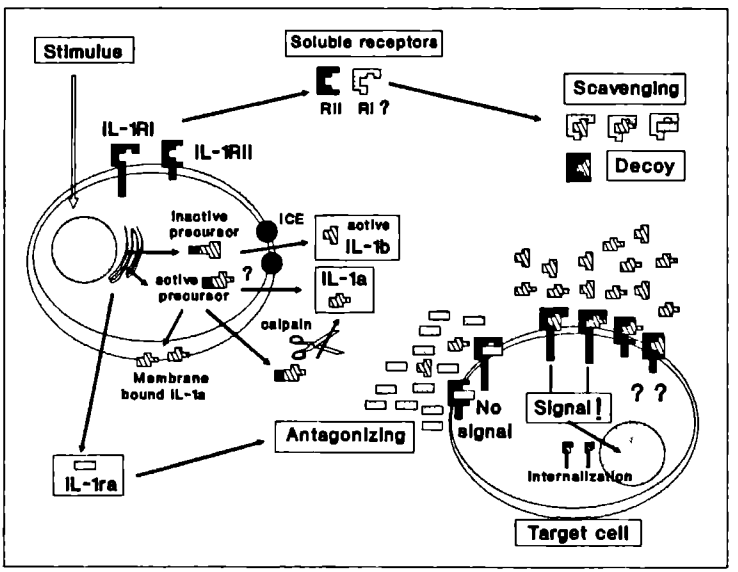

Figure 1. Levels to interfere with the IL-1 bioactivity.
The expression of mRNA of IL-1α and IL-1β can be increased by phorbol esters (PMA), lipopolysaccharide (LPS) and cytokines as IFN-gamma, TNF and IL-1, inducing an autocrine and paracrine loop. The mRNA expression can be decreased by prostaglandin-E_2 or cytokines as IL-4, IL-6, IL-10, TGFβ1, and probably IL-13. The biologically inactive IL-1β precursor is secreted and subsequently activated by the IL-1 converting enzyme (ICE). Specific inhibitors for ICE may prevent IL-1β secretion and activation. The mechanism of IL-1α secretion is still unknown. The bioactivity of secreted IL-1 can be controlled by different mechanisms: First, IL-1 can be scavenged by soluble IL-1 receptors. IL-1 receptor type II (RII) is a shedding product of PMNs with a high affinity for IL-1β. Second, IL-1 can be antagonized by the IL-1 receptor antagonist protein (IL-1ra). The expression of IL-1ra can be enhanced by TGFβ and IL-1. Third, the number of IL-1 receptors on the target cells can be reduced by IL-1, IL-2 and TGFβ or increased by IFN-gamma. Fourth, IL-1 can simply be counteracted by other cytokines e.g. IGF-1 on chondrocytes.

Several groups showed evidence that the IL-1:receptor complex is internalized and translocated into mitochondria and the nucleus [51]. This suggest that IL-1 bypasses the currently known signal transduction pathways and more directly exerts its activity on the cell metabolism. Computer analysis showed that the IL-1RI had a region with strong amino acid sequence homology with other virus associated nuclear factors [52]. Furthermore, it is shown that a synthetic IL-1ß fragment is internalized into the cytosol without receptor binding and that the IL-1 receptor is not necessary for IL-1 to exert an effect [53]. Modifications of the tertiary IL-1ß structure, point mutations and monoclonal antibody mapping demonstrated different regions for IL-1 receptor binding and the different biological activities [54-56].

The following cells express primarily the IL-1RI: chondrocytes, TH2 (subtype of T-cells), fibroblasts, keratinocyten, hepatocyten and endothelial cells; whereas type II is predominantly expressed on B-cells, TH1, neutrophils, bone marrow cells and macrophages.

1.3 Detection of IL-1: relationship to disease activity in RA.

In general, IL-1ß levels (several hundreds of pg/ml) in synovial fluids from RA patients are far greater than those found in osteoarthritis patients in comparative studies and are significantly increased compared to normal subjects. IL-1 is detected in synovial fluids of patients from different arthropathies: Rheumatoid arthritis, Psoriatic arthritis, Reiter's syndrome, Yersinia reactive arthritis, Osteoarthritis, Ankylosing spondyltitis, Gout, Traumatic arthritis, Lyme disease, but only Staphylococcal-related arthritis is reported to be IL-1 negative [57-63].

At the systemic level, IL-1 levels in peripheral blood plasma and serum are correlated to the disease activity of arthritis [64]; synovial fluid IL-1ß levels correlate positively with the amounts of C3a des Arg and IC in Psoriatic arthritis and with C3d in RA [65,66], indicating an active inflammatory process. Danis *et al.* showed that the peripheral blood monocytes from RA patients spontaneously express IL-1α and IL-1ß in culture [67]. Peripheral blood mononuclear cells spontaneously produced IL-1 when taken at the onset or during a recent relapse of rheumatoid arthritis [68]. Duff *et al.* could only detect IL-1α positive peripheral blood cells, having a dendritic morphology, using a direct Elispot technic [69], and there incidence correlated positively with the index of disease

activity in RA [70]. Furthermore, IL-1ß levels in serum but not in synovial fluid correlate positively with the pain score, erythrocyte sedimentation rate, Ritchie articular index, and histological changes and HLA-DR4 expression in the joint capsule in RA [71-74].

At the local level, synovial IL-1 levels correlates significantly with the numbers of PMNs in the joint but appeared to be unrelated to the total number of leucocytes in both RA and OA [74-76]. PMNs have a dual role, they synthesize IL-1α and IL-1ß and the IL-1 antagonist IL-1ra [77-79]. Malyak *et al.* showed that the synovial fluid PMNs contributed to the local IL-1ra levels [76]. This could explain the higher biological IL-1 levels in synovial lavage of sero negative (rheumatoid factor, RF) RA-patients compared to seropositive RA with the more severe inflammation.[3] Furthermore, this may explain the absence of correlation of synovial IL-1 levels with indices of systemic inflammation in RA-patients [80]. By antagonizing IL-1, PMNs can attribute to the remission of the disease. The interval to recover from Lyme disease is linked to the balance of [IL-1]/[IL-1ra] in the synovial fluid [81]. It would be intersting to consider the role of the PMN in Lyme disease as an 'anti-inflammatory' cell.

Immunohistochemistry showed IL-1α positive cells in the lining layer, interaggregate area, vascular endothelium, and lymphocyte aggregates of synovia. IL-1α is also detected in pannus tissue and chondrocytes adjacent to pannus [82]. IL-1ra is also present at the IL-1 positive spots although the incidence is smaller. Strangely enough, so far they couldn't find IL-1ß in synovium from normal subjects and neither in cartilage nor pannus of RA patients [83]. However, Farahat *et al.* showed that both IL-1α and IL-1ß are equally expressed by the synovial cells in the connective tissue area (number of IL-1 positive cells is 40%), perivascular area (50-55%), and the lining layer (80%) [84]. The number of IL-1 positive cells in the same areas is significantly smaller in OA-patients than in RA-patients.

In most RA-patients, IL-1ß but not IL-1α could be detected in synovial fluids. One possible explanation is that IL-1α is present in the cytoplasm or on cell membranes and IL-1α is only released after cell death. A likely explanantion, as it is shown that isolated synovial macrophages do express both IL-1 isotypes in culture with a more prolonged expression of IL-1α mRNA [67,85,86].

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[3] Plenary lecture JM DAYER, IAIS, Vienna, October 10-15, 1993.

Beuscher *et al.* [87] showed that maturation of macrophages coincided with a shift from IL-1ß into IL-1α producing cells in mice infected with *Yersinia enterocolitica*. Secondly, IL-1α could be neutralized by autoantibodies present in sera of rheumatoid arthritis patients [88-90].

Dayer-JM[3] found that the synovial fluid levels of IL-1 correlate good with the disease activity in osteoarthritis and that the synovial fluid TNFα levels correlate even beter than the IL-1 levels with the disease activity in RA. The serum IL-1ß levels correlate positively with serum TNFα levels [75,91], however, the IL-1 levels in synovial fluids are unrelated to the levels of TNFα, IL-2 or IFN-gamma.[3] Nevertheless, Brennan *et al.* found that the spontaneous IL-1 production could be blocked by anti-TNFα antibodies in a culture of synovial lavage cells and isolated synoviocytes obtained from RA but not from OA [8.92]. They also analysed cellular composition (macrophages and T-cells) in the synovial lavage. Interestingly, higher IL-1 production are shown in synovial lavage with T-cell:macrophage ratio's greater than 1 whereas the TNFα production is inversely related to the number of T-cells. Yanni *et al.* found that synovia from RA-patients characterized by multiple focal lymphoid aggregates of mononuclear cells, show higher IL-1 production than rheumatoid synovia with a disperse mononuclear cell infiltrate [93]. The infiltrating T-cells are probably TH1-cells as IL-2 and IFN-gamma are potent factors to prime fibroblasts [94] whereas the TH2 cell derived IL-4 and IL-10 are potent inhibitors of LPS-induced IL-1 production [95,96].

One of the features of RA is cartilage destruction and several reports suggested a link with IL-1 bioactivity. Saxne *et al.* reported that synovial fluids and some of the sera of RA patients moderately increased degradation of viable bovine nasal septum cartilage and markedly inhibited chondrocyte proteoglycan (PG) synthesis [97]. Hollander *et al.* showed by immunoabsorbance in different patients that either IL-1α, IL-1ß, or TNFα were involved in the accelerated cartilage loss although the respective recombinant cytokines were not as effective in cartilage explant cultures [98,99]. IL-1 was identified as the factor present in conditioned medium of RA-synovial membrane derived cells (macrophages and fibroblasts) to cause proteoglycan degradation in living cartilage [100] and not oxygen-radicals [101]. However, the concentrations of PG remnants in the synovial fluid or the inhibition of PG synthesis and degradation on cartilage explants could not be correlated with the synovial fluid IL-1ß concentrations [74]. It must be emphasized that Saxne *et al.* measured

immunoreactive IL-1 instead of the concentrations of bioactive IL-1. The cytokines leukemia inhibitory factor (LIF) and basic fibroblast frowth factor (bFGF) are known to synergize with IL-1 to cause cartilage destruction [102, 103]. However, a novel yet unidentified synovial fibroblast derived factor, chondrocyte activating factor (CAF), is implicated in the enhanced chondrocyte mediated cartilage breakdown [104]. CAF activity is distinct from IL-1, TNF, bFGF, IL-6, and IL-8.

1.4 Evidence of IL-1 production in experimental models of arthritis.

In the collagen-induced arthritis (CIA) model in rats, the spontaneous and LPS-stimulated IL-1 release is increased at the onset of arthritis and thereafter [105]. Interestingly, the lymphocytes from these rats also spontaneously produce high amounts of IL-2. Increased levels of bioactive IL-1 in the soluble compartment of bone marrow from tibia and femur precede the clinical onset of both adjuvant arthritis (AA) and CIA, and remains elevated during the course of arthritis [106]. The elevated levels of IL-1 coincide with a shift in the bone marrow cells from a predominant lymphoid population into a mixed population of myeloid cells and PMNs. At the local level, the course of IL-1 production as found in whole joint extracts duplicated the course of footpad swelling, and is elevated for weeks in CIA in mice [107]. In contrast to the rise of IL-1 in the bone-marrow, the peritoneal macrophages from AA-rats spontaneously produce reduced levels of IL-1 compared to normal rats although equivalent amounts are detected after LPS-stimulation [105]. It is unclear whether the reduction in IL-1 levels found in the culture supernatant of peritoneal macrophages was due to the presence of IL-1 inhibitors (TGFß, IL-1ra).

Peritoneal macrophages from a rat strain sensitive for Streptococcal cell wall (SCW)-induced arthritis, produce more IL-1 than macrophages obtained from a resistant strain after SCW-peptidoglycan-polysaccharide stimulation and this is not due to differences in IL-1ra production [108]. However, a causal relationship between the IL-1 release and the development of arthritis was not implicated by these studies. Bioactive IL-1 (8-16 ng/ml) are also found in joint washes (1 ml saline) of inflamed joints at day 1 of antigen (ovalbumin)-induced arthritis in rabbits followed by a rapid decline thereafter with an IL-1 level of

approximately 25-50 pg/ml at day 4 [109]. The cellular origin of IL-1 is not elucidated. Leukocytes harvested from the synovial cavity produced high levels of IL-1 (11.9 ng/10^6 cells/24 h) at day 7 and only small amounts at day 1 and 21. Furthermore, explants of synovial lining of normal joints and inflamed joints at day 1 of arthritis produced about 1 ng/100 mg tissue/96 h, and levels increased upto 7 and 16 ng/100 mg tissue/96h at day 7 and 21, respectively. This indicate that PMNs, the predominant infiltrating cell at day 1 of arthritis, can not be the source of IL-1 present in in the synovial fluid at day 1. Furthermore, it suggest that the mononuclear cells are responsible for the IL-1 production.

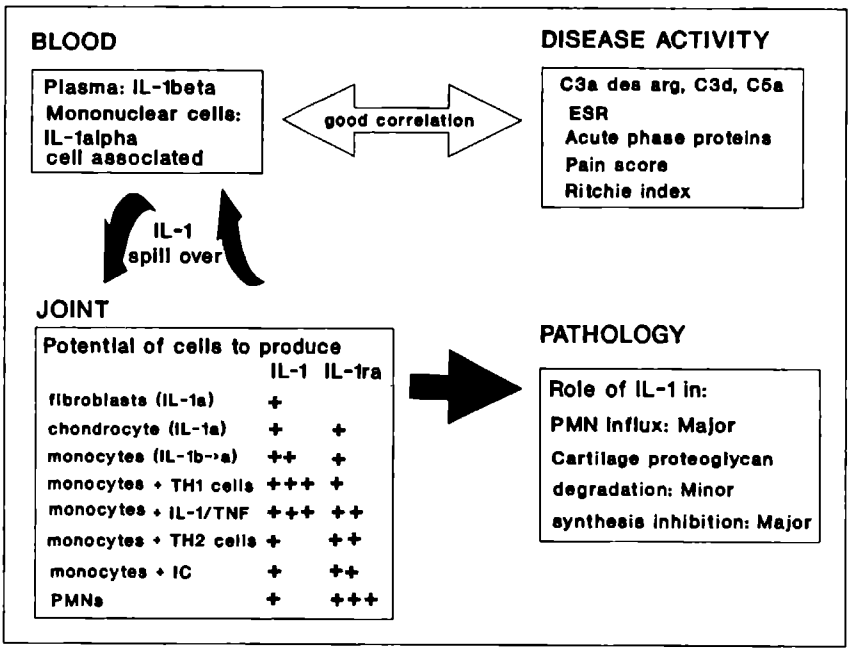

Figure 2. Role of IL-1 in arthritis.
Circulating IL-1 levels correlates with parameters of disease activity, and IL-1 is implicated in some of the pathological changes in the arthritic joint. At the local level, numerous cells are capable of IL-1 synthesis. The extent of IL-1 production may depend on the arthritogenic trigger, number and composition of the infiltrating inflammatory cells, and the production of the IL-1 receptor antagonist protein (IL-1ra). Spill-over of IL-1 from the circulation may affect the arthritic process in the joint. Abbreviations: ESR, erythrocyte sedimentation rate; TH1, T-helper cell type 1; IC, immunecomplex; PG, proteoglycan.

The fact that IL-1 could not be found in the synovial fluid after day 1 can be explained by the fact that IL-1 is not released into the cavity, masked by inhibitors, or under the stringent suppressive influence of mediators *in situ*. It is recently known that both monocytes and PMNs are capable of producing the IL-1 isotypes and the IL-1 receptor antagonist [77-79].

In the mouse model of antigen-induced arthritis, IL-1 levels are assessed in a 1 h culture of articular specimen. We showed a rapid onset of IL-1 bioactivity within the first hours of AIA, high levels were found in explants taken at 6 and 24 hours (30 pg/10 mg tissue/1 h), and declined thereafter [110]. In the immune-complex mediated arthritis (ICA) model in mice the IL-1 production declined more rapidly and was absent at 24 h of arthritis. The latter may be related to the low antigen amount; 2 μg mBSA, a dose equivalent to the antigen dose used in ICA, also induced a more transient IL-1 kinetic in AIA [111]. ICA induced in neutropenic mice demonstrated the same IL-1 production suggesting that PMNs play no role in this.

The above findings have important consequences for timing of IL-1 blocking *in vivo* as IL-1 production increases prior or directly after onset of arthritis. As seen in AA, IL-1 production will often be limited to certain areas within the animal. For example, peritoneal macrophages of MRL-lpr autoimmune mouse do not spontaneously produce IL-1 during arthritis, although they expressed increased levels after LPS-stimulation identical to control BALB/c mouse [112].

Furthermore, these studies suggest that the macrophages are possibly the prime candidates of IL-1 production during the course of arthritis both at the local and at remote sites. In a recent publication, cytoplasmic IL-1α is detected in macrophages of the synovial lining and sublining of experimental Staphylococcus aureus arthritis in mice [113].

1.5 Framework of this study.

It requires the fullfillment of several criteria to decide that a cytokine plays a major role in arthritis: first, the cytokine must cause or contribute to the type of pathology observed in arthritis; second, to show that there is sufficient cytokine present to cause the postulated effects; and third, to show that agents which selectively block the action of the cytokine can affect arthritis.

In **chapter 2** the effect of murine recombinant IL-1α and IL-1β on the proteoglycan metabolism in murine articular cartilage was tested. Patellae were incubated with IL-1 and the chondrocyte proteoglycan synthesis and breakdown were measured with [^{35}S]-sulfate radiolabel incorporation. The marked effects of IL-1 on the proteoglycan metabolism *in vitro* was also evaluated *in vivo*. In **chapter 3** it is shown that intra-articular injections of IL-1 into murine knee joints caused a prolonged suppression of the proteoglycan synthesis and a transient acceleration of proteoglycan degradation. Repeated IL-1 injections caused an arthritic insult and the mechanism of cartilage recovery was further investigated by detailed of proteoglycan synthesis and degradation in **chapter 4.**

To prove that IL-1 played a role in the arthritic process mice were treated with neutralizing rabbit anti-IL-1 polyclonal antibodies. In **chapter 5** the effects of the anti-IL-1 pre-treatment on antigen-induced arthritis are described. Anti-IL-1 treatment normalization the proteoglycan synthesis but had no effect on the onset of joint inflammation. In **chapter 6** it is shown that anti-IL-1 treatment had no effect on the accelerated breakdown in murine antigen-induced arthritis. In the comparative study of **chapter 7**, the *in vivo* roles of IL-1, IL-6 and TNF were examined in antigen-induced arthritis and the non-immunologically mediated, zymosan-induced arthritis, an acute inflammatory model in mice. Both IL-6 and TNF were not involved in the suppression of the proteoglycan synthesis, however, IL-1 was responsible for the suppression in these models. The long-term effect of the IL-1 blockade on inflammation and cartilage proteoglycan matrix was studied in antigen-induced arthritis.

The pro-inflammatory property of IL-1 was investigated and described in **chapter 8**. Intraarticular or systemic injections of IL-1 exacerbated the smouldering joint inflammation in the chronic phase of antigen-induced arthritis. In **chapter 9** the role of IL-1 was studied using anti-IL-1 antibodies in the T-cell mediated, antigen-induced exacerbations of AIA. In this phase, blocking IL-1 dampened the inflammation and ameliorated cartilage destruction.

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CHAPTER 2

EFFECT OF MURINE RECOMBINANT INTERLEUKIN-1 ON INTACT HOMOLOGOUS ARTICULAR CARTILAGE: A QUANTITATIVE AND AUTORADIOGRAPHIC STUDY

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Effects of murine recombinant interleukin 1 on intact homologous articular cartilage: a quantitative and autoradiographic study

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SUMMARY Murine recombinant interleukin 1 (IL1) was tested for its ability to affect intact murine articular cartilage IL1 caused enhanced proteoglycan degradation and severe inhibition of chondrocyte synthetic function at a concentration of 3 U/ml (100 pg/ml) Inhibition of proteoglycan synthesis appeared to be delayed in onset but occurred consistently after 24 hours Pulse chase experiments made it clear that proteoglycan degradation and inhibition of proteoglycan synthesis are two distinct actions of IL1 No indications were obtained for selective degradation of either newly synthesised or processed proteoglycan Moreover, chondrocyte synthetic activity appeared to be inhibited uniformly throughout the cartilage matrix, i e , no evidence was found for selective suppression of cells in certain regions IL1 uptake measurement in the cartilage, using [^{125}I]IL1, yielded a partition coefficient far below 1, and autoradiography demonstrated a faint but even distribution within the cartilage matrix The coordinated induction of enhanced breakdown of proteoglycan and inhibition of proteoglycan synthesis, with such low concentrations of IL1 reaching the chondrocytes, underlines the impressive destructive potential of IL1

Key words proteoglycan synthesis, cytokines, murine cartilage

The term interleukin 1 (IL1) was originally introduced to describe a 17 kilodalton protein, secreted by activated cells of the macrophage/monocyte lineage, that augmented T cell proliferation to mitogens and antigens [1] This property was formerly known as lymphocyte activating factor (LAF) activity It has now become evident that IL1 can affect multiple cell types and functions For instance, IL1 may mediate fever, hepatic secretion of acute phase proteins, lymphocyte chemotaxis, fibroblast proliferation, and alterations in bone metabolism [2] Recent work using recombinant IL1 has confirmed that these several biological activities reside within one molecule [3] Earlier studies had suggested that IL1 is also involved in cartilage destruction in arthritic joints Coculture of cartilage and synovial tissue resulted in severe chondrocyte mediated breakdown of cartilage proteoglycans The substance responsible for this effect was termed catabolin [4] Similar effects were described for mononuclear cell factor,[5] a product of stimulated human blood monocytes When purified to homogeneity, catabolin and mononuclear cell factor were shown to have properties identical to those of IL1 [6 7] It is now known that IL1 stimulates the release of metalloproteinases from chondrocytes [8–10] Interestingly, IL1-like activity has been identified in synovial fluid from patients with various types of joint disease [11 12] Moreover, raised levels of cartilage derived proteases have been detected in cartilage specimens from patients with osteoarthritis [13 14]

In the present study we describe the effects of murine recombinant IL1 on chondrocyte metabolism, using anatomically intact homologous articular cartilage—the murine patella Apart from an enhancing effect on proteoglycan breakdown, IL1 was shown to inhibit severely chondrocyte proteoglycan synthesis as measured by ^{35}S incorporation

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Autoradiography was performed to detect potential variability in IL1 sensitivity of chondrocytes in various regions Finally, IL1 was radiolabelled to determine its partition in intact cartilage

Materials and methods

PREPARATION OF IL1

The published amino acid sequence of murine IL1α was used to synthesise a cDNA fragment and this was expressed in *Escherichia coli* The protein consisted of the carboxy terminal amino acids 115 to 270 of the IL1 propeptide The molecule was purified from inclusion bodies and refolded into the biologically active form (G Daumy to be published) Cyanogen bromide fragmentation was used for structural confirmation Biological activity was verified in multiple assays (Otterness *et al* in preparation) In the LAF assay the IL1 was found to give 1 unit activity consistently in the 10–40 pg/ml range Screening for endotoxin activity (Limulus assay) was negative up to a concentration of 100 μg/ml IL1 The recombinant IL1 preparation was stored at −20°C and showed consistent LAF activity over the period studied Experiments were performed with two batches of IL1 prepared on different occasions Effects on chondrocyte synthesis and cartilage breakdown were completely similar

LAF ACTIVITY

Murine thymocytes from mice aged 6–7 weeks were cultured for four days in the presence of 1 μg/ml phytohaemagglutinin and various IL1 concentrations Thymocytes were cultured at a concentration of 10^7/ml in 200 μl wells and [^{3}H]thymidine was added (37 kBq/well) during the last day One unit of LAF activity was defined as the IL1 concentration giving half the plateau of IL1 induced comitogenic thymocyte proliferation

CARTILAGE ASSAY

Whole murine patellae were isolated from C57Bl mice leaving the patella embedded in a minimal amount of surrounding tissue and used to measure chondrocyte metabolism as described previously [15 16] In brief, patella specimens were cultured in RPMI-5% fetal calf serum (Flow) under 5% CO_2 with or without IL1 Culture medium was refreshed every day To determine chondrocyte proteoglycan synthesis the specimens were subsequently pulsed with 740 kBq [^{35}S]sulphate/ml for three hours After washing, fixation in formalin (10%), and decalcification in formic acid (5%) the patella could easily be punched out of the adjacent tissue The ^{35}S content of each patella which is a reliable measure of the [^{35}S]glycosaminoglycan content [17 18] was measured by liquid scintillation counting

To measure the potential release of labelled proteoglycan into the medium 100 μl aliquots of the three hour [^{35}S]sulphate pulse medium of control and IL1 incubations were treated with 1% cetylpyridinium chloride Non-labelled proteoglycan was added as a carrier After two hours at 37°C the precipitate was spun down at 10 000 g for 30 minutes and the pellet was washed five times with 0 1% cetylpyridinium chloride to remove adherent [^{35}S]sulphate [17] To correct for loss of [^{35}S]proteoglycan during this procedure the following standard was included in the experiment [^{35}S]proteoglycan (2000 cpm) from bovine cartilage supplemented with 74 kBq [^{35}S]sulphate

To determine IL1 mediated degradation the patellae were prelabelled with [^{35}S]sulphate (6–24 h) before IL1 exposure After the culture period with IL1 the amount of ^{35}S retained in the patellar cartilage was measured and expressed as a percentage of the ^{35}S content of control cartilage cultured without IL1

To enable comparison of IL1 effects on newly synthesised and more extensively processed proteoglycans mice were injected intraperitoneally with 74 kBq/g body weight and labelled patellae were isolated at days 1 2 and 4 The right patella was cultured in the presence of IL1 for 24 hours and the left patella was cultured without IL1

The IL1 effect was always expressed in comparison with the value for the contralateral joint of the same animal This approach minimises variation due to interindividual difference in ^{35}S incorporation [15]

AUTORADIOGRAPHY

Patella specimens were fixed and decalcified as described above After histological processing 6 μm sections were prepared and mounted on gelatin coated slides These were dipped in K_5 emulsion (Ilford, Basildon Essex England) and exposed for three to five weeks After this period the slides were developed and stained with haematoxylin and eosin

CARTILAGE HISTOLOGY

Patella sections were stained with alcian blue for 24 hours The dye was solved in an acetate buffer (0 025 M, pH 5 4) at a concentration of 0 06%, in the presence of 0 3 M $MgCl_2$ Decreased staining of the cartilage layer reflects loss of proteoglycan Similar results were obtained with Safranin O or Giemsa staining

UPTAKE OF RADIOLABELLED IL1

^{125}I labelling of IL1 was performed by the method of

Bolton-Hunter Iodinated IL1 was separated from free ^{125}I by Sephadex G 25 fractionation The labelled preparation retained full biological activity, and the specific activity was approximately 37 kBq/μg

Patellae were incubated with [^{125}I]IL1 in phosphate buffered saline (pH 7 4) for various time periods at 37°C At the end of the incubation the specimens were rinsed once, blotted dry, and fixed in 10 ml of 2 5% glutaraldehyde or formalin (10%)/ethanol for 30 minutes This was followed by decalcification in 5% formic acid overnight After a short fixation period the whole cartilage layer can easily be stripped from the underlying bone,[17] and the amount of [^{125}I]IL1 present in the cartilage can be counted The procedure of fixation of cartilage associated protein allows the release of free ^{125}I from the tissue slight contamination of labelled protein solutions with free ^{125}I would otherwise introduce significant errors owing to the relatively high uptake of free ^{125}I compared with that of poorly penetrating proteins The uptake of [^{125}I]IL1 is expressed as a partition coefficient, which is the concentration in the cartilage divided by the concentration in the incubation medium To obtain the partition volume of the patellar cartilage strip the cumulative wet and dry weights of 10 specimens were determined The fluid phase of one patella strip was approximately 0 05 μl

Results

INHIBITION OF CHONDROCYTE PROTEOGLYCAN SYNTHESIS

Initially, whole patellae were incubated with various concentrations of murine recombinant IL1 for 48 hours, followed by a three hour pulse with [^{35}S]sulphate, to define the effective concentration range of IL1 IL1 caused a dose dependent inhibition of [^{35}S]proteoglycan synthesis (Table 1) Concentrations of IL1 of 0 1 U/ml or below were essentially without effect A plateau of maximal inhibition was reached at IL1 concentrations of 3 U/ml and higher In multiple experiments inhibition ranged between 50 and 65% with an IL1 concentration of 3 U/ml Control incubations with recombinant IL2 (Amersham, England), tested in doses up to 30 U/ml, failed to inhibit proteoglycan synthesis To verify that we were really measuring IL1 mediated inhibition of synthesis and not enhanced breakdown of new proteoglycan synthesised at the same rate we checked the culture supernatant for labelled proteoglycan fragments The amount released during the three hour ^{35}S pulse period was less than 10% of the amount incorporated in the cartilage for both the control incubation and after 48 hours of IL1 exposure, and was therefore negligible

Next, the effect on sulphate incorporation of time of exposure to IL1 was explored After 24 hours IL1 exposure the suppression of chondrocyte synthesis (^{35}S incorporation) was variable (5–20%), only rarely reaching statistical significance Exposure of patellar cartilage to IL 1 for 48 hours consistently resulted in extensive inhibition of proteoglycan synthesis (Table 2) This effect seems not to depend upon exposure of the cartilage to IL1 for 48 hours as incubation with IL1 for 24 hours followed by a

Table 1 *Dose dependent effect of IL1 on ^{35}S incorporation*

IL1 concentration (U/ml)*	*^{35}S incorporation† (cpm/patella)*	*Inhibition (%)*
—	357 (33)	—
0 03	345 (19)	3
0 1	309 (25)	13
0 3	288 (34)	19
1 0	227 (32)	36
3 0	161 (39)	55
10 0	125 (37)	65
30 0	139 (31)	61

Patellae were incubated for 48 hours
*Values are U/ml based on thymocyte proliferation
†Values are the mean (SD) of six cartilage specimens

Table 2 *Time dependence of IL1 effect on synthesis*

Time (h)	*IL1 (U/ml)*	*^{35}S incorporation† (cpm/patella)*	*Inhibition (%)*
24	—	737 (163)	—
24	3	575 (86)	22
48	—	626 (95)	—
48	3	288 (46)	54*

Patellae were exposed to IL1 for 24 or 48 hours followed by a three hour pulse with [^{35}S]sulphate
*p<0 001 compared with 24 hour value (Mann Whitney)
†Values are the mean (SD) of six cartilage specimens

Table 3 *Time lag in IL1 effect on synthesis*

Incubation with IL1 (h)	*Length of chase (h)*	*^{35}S incorporation* (cpm/patella)*	*Inhibition (%)*	*p Value†*
—	48	540 (67)	—	
3	45	452 (35)	16	0 001
24	24	294 (52)	46	NS
48	—	278 (35)	49	

Patellae were incubated with IL1 (3 U/ml) for various periods, followed by a chase without IL1 At the end of the incubation patellae were pulsed with [^{35}S]sulphate for three hours
*Values are the mean (SD) of six cartilage specimens
†p Values compared with 48 hour IL1 exposure (Mann Whitney)

24 hour incubation with medium only led to the same total effect (Table 3). These data indicate that IL1 mediated suppression of chondrocyte proteoglycan synthesis is delayed in onset. We never observed a suppression exceeding 25% at 24 hours, even with IL1 concentrations up to 100 U/ml. This indicates that slow diffusion of IL1 into the cartilage, reaching a sufficient level of IL1 near the chondrocyte in a retarded fashion, cannot be the reason for this time lag. We also looked for selective effects of IL1 on chondrocyte incorporation of [^{35}S]sulphate in different regions of the patella by performing autoradiography on semiserial patellar sections of cultured specimens. Uniform labelling of the cartilage layer was obtained after 48 hours' culture and three hours' [^{35}S]sulphate exposure (Fig. 1a). When the patellae were cultured in the presence of IL1, clear cut suppression of labelling intensity was observed (Fig. 1b). No evidence was obtained for a differential sensitivity to IL1 when the central or marginal regions, or the chondrocytes in superficial and deeper layers, were compared.

DEGRADATION OF [^{35}S]PROTEOGLYCANS

Patellae prelabelled with [^{35}S]sulphate for six hours were cultured with or without IL1, and the amount of ^{35}S retained in the patellar cartilage was determined. Significantly less ^{35}S was retained in cartilage cultured in the presence of IL1 (Table 4), indicating that proteoglycan degradation was enhanced. The action of IL1 was not influenced by the presence of fetal calf serum in the medium (Table 4). When larger concentrations of IL1 (up to 100 U/ml) were used, only slightly higher effects on breakdown were seen; not always reaching statistical significance compared with effects obtained with 3 U/ml.

To investigate whether there might be a preferential effect of IL1 on newly synthesised proteoglycan compared with older, more fully processed proteoglycan we carried out the following experiment. Effects of IL1 were compared on cartilage with variably processed proteoglycans, and for optimal physiological processing of labelled proteoglycans labelling was performed in vivo. From earlier studies it was known that the amount of [^{35}S]proteoglycan in patellar cartilage, after a single injection of [^{35}S]sulphate, reaches a plateau between two and six hours and gradually decreases thereafter.[15] Therefore the effect of IL1 on patellar cartilage isolated on days 1, 2, or 4 after [^{35}S]sulphate injection was determined (Table 5). Patellae taken at day 1 or 2 and cultured for 24 hours in the presence of IL1 both contained significantly less ^{35}S than those cultured in the absence of IL1. The enhancement of IL1 mediated breakdown was of the same order of magnitude for days 1 and 2. A similar result was found when the comparison was made between days 1 and 4. We repeated this experiment

Table 4 *IL1 mediated breakdown*

^{35}S Labelling (h)*	*IL1 (U/ml)*	*Fetal calf serum (%)*	*^{35}S content†*	
			cpm/patella	*%‡*
6	—	—	1047 (179)	—
6	3	—	775 (151)	74
6	—	5	1060 (244)	—
6	3	5	748 (110)	71
24	—	5	1897 (183)	—
24	3	5	1367 (189)	72

*Patellae were prelabelled with [^{35}S]sulphate for either six or 24 hours, followed by incubation for 24 hours with or without IL1.
†Values are the mean (SD) of six cartilage specimens.
‡Compared with its proper control.

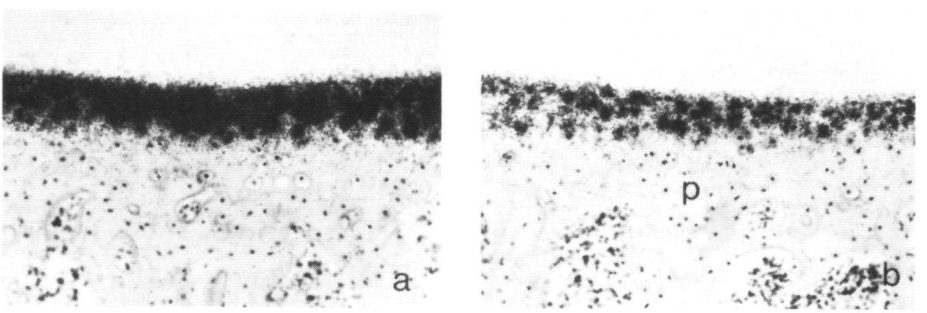

Fig. 1 *Autoradiography of patellar cartilage after 48 hours' exposure to (a) medium or to (b) 3 U IL1/ml, followed by a three hour pulse with [^{35}S]sulphate. Note the distinct labelling in (a) and the uniformly diminished labelling above all cells after IL1 exposure in (b). p=patella.*

Table 5 *Comparison of the effect of IL1 on newly labelled and more processed proteoglycan*

*Experiment**	*Time (h)*	*IL1 (U/ml)*	*^{35}S content†*	
			cpm/patella	*%‡*
A	24	—	248 (63)	—
A	24	3	185 (39)	75
A	48	—	228 (63)	—
A	48	3	180 (42)	79
B	24	—	380 (108)	—
B	24	3	275 (59)	72
B	96	—	302 (76)	—
B	96	3	221 (47)	73

Patellae were isolated and cultured for 24 hours with or without IL1, one patella without and the contralateral patella of the same animal with IL1.
*Mice were given an injection with 1·85 (exp A) or 2·78 (exp B) MBq [^{35}S]sulphate.
†Values are the mean (SD) of cartilage specimens of seven mice.
‡Compared with its proper control.

three times, with identical results. Finally, to verify that these results were not an artefact of radiolabelling, patellae were cultured for two days and then stained histologically. IL1 induced clear depletion of proteoglycan in the metabolically active superficial layer of the patella (Fig. 2).

IL1 UPTAKE IN CARTILAGE

IL1 was radiolabelled to permit measurement of its effective concentration in cartilage. The labelled preparation retained full biological activity as tested in the cartilage and in the LAF assay. Patellae were incubated with [^{125}I]IL1 for various time periods. The uptake reached equilibrium within 30 minutes, but the partition coefficient of the anionic IL1 (pI 5) remained far below 1 (Table 6).

A partition coefficient of 0·2 indicates that the local concentration is about five times less than the IL1 concentration in the incubation medium. In contrast, the cationic protein amidated bovine serum albumin (aBSA; pI 8·5–9)[19 20] accumulated to a high extent in the cartilage and equilibrium was not reached even after four hours (Table 6).

Figure 3 depicts the autoradiography of IL1 and aBSA partition. In contrast with the high affinity of aBSA for the patellar cartilage (Fig. 3b), IL1 was predominantly taken up by the surrounding tissue (Fig. 3a). Higher magnification of the cartilage (Figs 3c and d) showed that most of the IL1 radiolabel was present as a superficial lining layer, and that the quantitative measurement of the partition (Table 6) strongly overestimated the actual concentration within the matrix. If, after [^{125}I]IL1 exposure, the patella specimens were washed with saline and then prepared for autoradiography, labelling was no longer detectable, either at the cartilage surface or within the matrix. The label present within the matrix showed a diffuse pattern over the whole cartilage area.

OTHER EFFECTS OF MURINE RECOMBINANT IL1

Murine IL1 (pI 5) was examined in a number of

Table 6 *IL1 uptake in patellar cartilage*

Incubation time (min)	*Partition coefficient**	
	IL1	*aBSA†*
10	0·08	3·8
30	0·15	ND†
60	0·16	10·4
120	0·14	20·2
240	0·16	33·7

*The partition coefficient is defined as the concentration in the cartilage divided by the concentration in the surrounding fluid.
†aBSA=amidated bovine serum albumin; ND=not done.

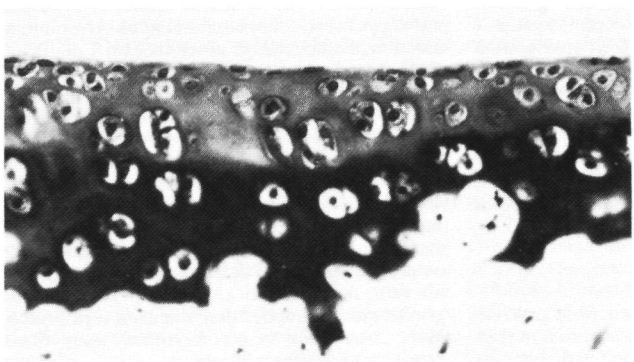

Fig. 2 *Staining of the patellar cartilage after 48 hours' IL1 exposure. Note the distinct loss of staining in the superficial layer, which is the metabolically active part of the cartilage (see Fig. 1). Halo formation around chondrocytes was not evident.*

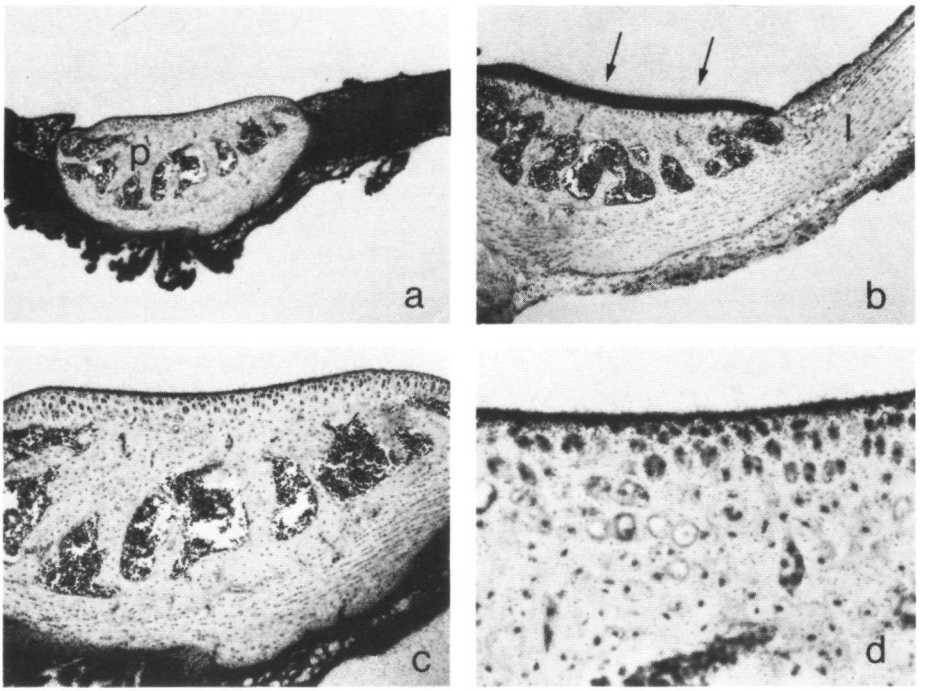

Fig. 3 *Autoradiography, four hours after incubation of patellae with (a, c, d) [^{125}I]IL1 or with (b) amidated bovine serum albumin ([^{125}I]aBSA). (a) IL1 is predominantly present in the surrounding connective tissue and ligament structures. (b) The cationic protein aBSA shows a high affinity for the patellar cartilage (arrows) and deeply penetrates the matrix. (c) and (d) are at higher magnification than (a) to show that IL1 is hardly present within the cartilage matrix. p=patella; l=ligament.*

other assays to compare its activity with that of native IL1. In vivo, in the mouse, we found it to induce fever, leucocytosis, i.e., elevation of granulocyte numbers, and acute phase reactant synthesis (serum amyloid A). In vitro it induced proliferation of thymocytes in the LAF assay. In the latter assay the IL1 was found to give 1 unit of activity consistently in the 10–40 pg/ml range. Preliminary experiments further indicated that the IL1 preparation also caused cartilage breakdown and proteoglycan synthesis inhibition in vivo upon intra-articular injection in knee joints (manuscript in preparation).

Discussion

The present study demonstrated that murine recombinant IL1 not only enhanced proteoglycan breakdown but also extensively inhibited chondrocyte proteoglycan synthesis in *intact* murine articular cartilage. As a control the lymphokine IL2 was without effect. The combined effects of enhanced proteoglycan breakdown and inhibition of restoration underline the destructive potential of IL1 in disease states characterised by IL1 overproduction.

IL1 mediated loss of proteoglycans from the cartilage matrix does not seem to be a selective process. Newly synthesised proteoglycan and proteoglycan processed for four days are affected equally (Table 5). Earlier studies with catabolin yielded similar results. Release of newly synthesised (labelled) proteoglycans and unlabelled proteoglycans from catabolin treated cartilage followed the same pattern.[21] These data are consistent with free diffusion of catabolic enzymes away from the chondrocyte. It implies that stimulation of proteoglycan breakdown is not dependent upon tissue proximity to the chondrocyte.

Upon stimulation with IL1 chondrocytes have been shown to release metalloproteinases capable of degrading both collagen and proteoglycan [8 22] Enzymes found in the medium of IL1 treated cartilage cultures are in the latent form and must be activated before they can be enzymatically assayed [22] This suggests that IL1 stimulated the release of latent metalloproteinases from chondrocytes and that a proportion of the enzyme is activated in situ in the matrix Suggestive evidence for this mechanism stems from detailed analysis of cartilage proteoglycans after stimulation with IL1 [23]

In addition to proteoglycan degradation, IL1 induced extensive inhibition of chondrocyte proteoglycan synthesis This inhibition appears to be retarded in onset Effects were variable and not impressive at 24 hours but very significant at 48 hours A similar delay in onset of action was described for catabolin [24] So far, the second messenger involved in the effect of IL1 on synthesis has not been elucidated A recent report, however, clearly demonstrated the presence of specific IL1 receptors on chondrocytes [25] One could argue that the time lag in IL1 action on proteoglycan synthesis is compatible with the suggestion that IL 1 may stimulate new synthesis of degradative enzymes and that the apparent inhibition seen at 48 hours is therefore due to a high level of proteoglycan degrading enzymes and more severe breakdown of new proteoglycans synthesised at a normal, i e , unchanged, rate No evidence has been found however, for a faster, more extensive degradation of newly synthesised proteoglycan during the short ^{35}S pulse after long term IL1 exposure Tyler did not observe enhanced amounts of ^{35}S labelled proteoglycan fragments in the culture medium of such IL1 treated and briefly ^{35}S pulsed cartilage,[24] and in this study we obtained similar results Moreover, our pulse chase experiment made it clear that 24 hour IL1 exposure, followed by a chase without IL1, resulted in the same extent of inhibition of synthesis (Table 3) This makes it highly unlikely that the inhibition of synthesis is due to enhanced breakdown as it is known that proteoglycan breakdown ceases within 24 hours of removal of IL1 [21] Taken together, our data clearly indicate that IL1 has two effects on cartilage firstly, enhanced degradation of proteoglycan, secondly, extensive inhibition of proteoglycan synthesis via an as yet unknown mechanism

IL1 has to penetrate the cartilage before it is active Uptake of a protein in hyaline cartilage depends on both its size and charge Earlier studies from our group showed that proteins with an isoelectric point (pI) above 8 5 have a high affinity for the negatively charged cartilage and easily penetrate and accumulate in the dense matrix [19 20 26-28] The data obtained for IL1 uptake in the present study indicate a partition coefficient of 0 2, pointing to concentration of mediator inside the cartilage five times lower than in the surrounding fluid This value is consistent with published results for cartilage distribution of other anionic proteins of similar molecular weight [29] Our autoradiography made it clear however, that most of the radiolabel was located at the surface and that the actual II 1 concentration within the matrix was far less These observations stress the need for autoradiographic control of radiolabel studies The high amount of label at the surface is just a consequence of the technique used and does not reflect IL1 binding at that site In uptake studies patella specimens are not washed and surface binding of attached label does occur after fixation When washed with saline before fixation, this label is not detectable anymore Another relevant issue in uptake studies with labelled proteins is the potential change in behaviour due to labelling We chose the mild iodination according to Bolton-Hunter and checked that the [^{125}I]IL1 retained its full biological activity in our cartilage assay Moreover, at most one ^{125}I molecule is introduced per IL1 molecule, making a behavioural change highly unlikely Loss of activity in our cartilage assay was clear cut after choramine-T iodination of IL1 (data not shown) Earlier observations from our laboratory have indicated that penetration of anionic proteins increases when the cartilage matrix is depleted of proteoglycans [20 26] This indicates that IL1 penetration and interaction with chondrocytes could be expected to increase in already damaged cartilage of an inflamed joint Thus IL1 effects may be slightly higher in fibrous cartilage than in dense hyaline cartilage owing to penetration differences Although the two forms of this mediator, IL1α (pI 5) and IL1β (pI 7–8), differ considerably in isoelectric point, neither form has a pI above 8 5, and even IL1β would not be expected to accumulate in hyaline cartilage [28] Slight differences in potency between IL1α and IL1β, as noted in cartilage assays,[7] are not likely to be caused by disparity in uptake, and probably relate more to differences in IL1 chondrocyte (receptor) interaction

In experimental models of murine joint inflammation, such as zymosan or antigen induced arthritis, chondrocyte proteoglycan synthesis was already significantly suppressed at day 1 [16 30] From the present kinetic data it seems unlikely that IL1 is directly responsible for this early suppression Diffusion of mediators of acute inflammation such as hydrogen peroxide[31] or neutrophil enzymes such as elastase[32 33] may play a part in this early phase After the first day IL1 seems a likely candidate for

the continuing suppression of proteoglycan synthesis The suppression found in the models of experimental arthritis was of the same order of magnitude (50–60%) as that obtained with IL1 in vitro In vivo it was observed that there was a higher vulnerability of chondrocytes in certain regions of the articular cartilage [30] For instance, suppression of sulphate incorporation was more pronounced in chondrocytes of the central area of the patellar cartilage than in the margins [30] Evidence for an enhanced susceptibility of particular chondrocytes to IL1 effects was not observed in vitro in the present study This could indicate that IL1 is not the mediator of the cartilage damage seen in these models In vivo however load bearing may give rise to regional differences Moreover diffusion of proteolytic inhibitors or other protective factors from the margins supplied in vivo, may lead to more pronounced suppression in the central area

The importance of IL1 as a mediator of cartilage destruction in chronic destructive arthritis is yet to be determined IL1 activity has been detected in synovial fluid of patients with rheumatoid arthritis [11 12] Inhibitors are also present in high amounts however and measurement of consistent IL1 activity depends upon separation of IL1 and its inhibitors by gel chromatography [34 35] Some inhibitors appear only able to block the IL1 comitogenic assay [36 37] but recently an inhibitor has been described which blocked fibroblast collagenase production [38] and this may be an important regulator of tissue destruction IL1 must penetrate cartilage and escape from (local) inhibitors before it is active The recent observation that IL1 could induce joint inflammation and cartilage degradation [39–41] provides further evidence for its role as a key mediator Experiments with selective (synthetic) inhibitors of IL1 effects or IL1 synthesis and release are needed to elucidate this issue fully

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CHAPTER 3

EFFECT OF MURINE RECOMBINANT INTERLEUKIN-1 ON SYNOVIAL JOINTS IN MICE: MEASUREMENTS OF PATELLAR CARTILAGE METABOLISM AND JOINT INFLAMMATION

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Effects of murine recombinant interleukin 1 on synovial joints in mice: measurement of patellar cartilage metabolism and joint inflammation.

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Abstract

Murine recombinant interleukin 1 was injected intra-articularly into mice. It induced a clear effect on patellar cartilage within 24 hours. A low dose of interleukin 1 (1 ng) elicited a significant reduction in [^{35}S]sulphate incorporation (50%) into proteoglycans and an accelerated breakdown (twofold) of ^{35}S prelabelled proteoglycan. Proteoglycan breakdown returned to normal rates (≈10%/day) 48 hours after a single interleukin 1 injection. Recovery of proteoglycan synthesis was delayed up to 72 hours, however, which implies that repair of the depleted cartilage matrix is retarded. Interleukin 1 induced only minor joint inflammation, too slight to be held responsible for the strong suppression of proteoglycan synthesis. Vehement joint inflammation was found after repeated interleukin 1 injections. The plasma extravasation and massive infiltration and exudation of leucocytes, predominantly polymorphonuclear leucocytes, were not a mere summation of single interleukin 1 effects, but point to interleukin 1 induced local hypersensitivity. The cartilage matrices of patella and femur were heavily depleted. Measurement of the extent of loss of ^{35}S prelabelled proteoglycan and the prolonged inhibition of [^{35}S]sulphate incorporation indicate that both inhibition of proteoglycan synthesis and enhanced loss of proteoglycan contributed substantially to this depletion.

Interleukin 1 is a 17 kilodalton soluble unglycosylated protein, secreted by activated cells of mononuclear origin. Two forms have been described, interleukin 1α and interleukin 1ß, which are related proteins that bind to the same receptor and show roughly the same biological effects.[1-3] This ubiquitous mediator displays many inflammatory and immunoregulatory activities.[1-5] After systemic administration interleukin 1 gave rise, for example, to an acute phase response, enhanced immunoglobulin production, and protection against bacterial injury.[6-10] Apart from systemic effects, related to high concentrations of circulating interleukin 1 during inflammatory processes,[11] interleukin 1 may also play a more direct part in local inflammatory events. It has been shown that interleukin 1 injected into

the skin attracted neutrophils, leading to microvascular injury.[12-14] Moreover, interleukin 1 enhanced the binding of lymphocytes to endothelial cells and promoted chemotaxis of these cells.[15-20] Recombinant interleukin 1, injected directly into rabbit knee joints induced only minor inflammation,[21] but it was highly effective in causing an exacerbation of the arthritic process in a previously injured joint.[22] The important role of interleukin 1 in arthritis is further substantiated by the observation that it is produced by the arthritic synovium[23-24] and it can be detected in substantial amounts in synovial fluids.[25-29] Whether interleukin 1 is the predominant pathological messenger or only one of many effector cytokines in arthritis remains is still unknown.

In addition, interleukin 1 may contribute to connective tissue activation and cartilage destruction. In vitro studies have shown that interleukin 1 (catabolin) added to living explants of cartilage induces loss of matrix proteoglycans and inhibition of synthesis of proteoglycans.[30-37] In vivo studies with purified interleukin 1 showed significant loss of proteoglycans and inhibition of proteoglycan synthesis after intra-articular injection, and the presence of clear joint inflammation.[38-40] Recent studies with recombinant interleukin 1 disclosed only minor signs of inflammation. Nevertheless, proteoglycan loss was clearly shown and it was concluded that degradation of cartilage induced by interleukin 1 was unrelated to inflammation.[21 41] Effects on proteoglycan synthesis were not determined. We studied the impact of recombinant murine interleukin 1 after single and repeated injections in the murine knee joint. Special attention was paid to measurement of both the inflammation and two indices of cartilage destruction-proteoglycan loss and inhibition of proteoglycan synthesis.

Materials and Methods

ANIMALS

C57bl/10 mice aged 8-10 weeks at the start of the experiments were used. They were fed a standard diet and tabwater freely.

CHEMICALS

Recombinant interleukin 1α and interleukin 1ß were kindly supplied by Dr I G Otterness (Pfizer Central Research, Groton CT). Biological activity was verified by a lymphocyte activating factor assay. The interleukin 1 gave 1 unit activity consistently in the 10-40 pg/ml range. Screening for endotoxin activity (Limulus assay) was negative up to a concentration of 100 μg/ml. The recombinant interleukin 1 preparation was stored at -20°C and showed consistent lymphocyte activating factor activity over the period studied.

Zymosan A was obtained from Sigma (*Saccharomyces cerevisiae*, n° Z-4250). Zymosan (30 mg) was dissolved in 1 ml saline through heating up to 100°C twice, then sonicated to obtain a homogeneous suspension. Batches were stored at -20°C.

LYMPHOCYTE ACTIVATING FACTOR ACTIVITY

Murine thymocytes from mice, aged 6-7 weeks, were cultured for four days in the presence of 1 μg/ml phytohaemagglutinin and various interleukin 1 concentrations. Thymocytes were cultured at a concentration of 10^7/ml in 200 μl wells, and [^{3}H]thymidine was added (37 kBq/well) during the last day. One unit of lymphocyte activating factor activity was defined as the interleukin 1 concentration giving half the plateau of comitogenic thymocyte proliferation induced by interleukin 1.

INTRA-ARTICULAR INJECTIONS

Recombinant interleukin 1 or Zymosan was injected through thesuprapatellar ligament into the left knee joint space. In experiments investigating the proteoglycan metabolism the contralateral joint received an equal volume (6 μl) of saline. In the technetium uptake experiments only an incission into the skin was made in the contralateral knee. In these experiments a control group was included, in which saline was injected into the left knee joint. From the latter experiments it became clear that saline neither induced measurable joint inflammation nor influenced proteoglycan metabolism.

^{99m}Tc UPTAKE MEASUREMENTS

Joint inflammation was determined by measurements of ^{99m}Tc pertechnetate uptake in the knee joints.[42] Briefly, animals were sedated by intraperitoneal administration of 4.5% chloral hydrate, 0.1 ml/10 mg of body weight. Approximately 370 kBq ^{99m}Tc in 0.2 ml saline was injected subcutaneously in the neck region. After 15 minutes the accumulation of isotope in the knee, owing to increased blood flow and tissue swelling, was determined by external gamma counting. The severity of inflammation was expressed as the ratio of the ^{99m}Tc uptake in the left (zymosan or interleukin 1 injected) over that in the right knee joint.

BREAKDOWN OF [^{35}S]PROTEOGLYCANS

To study in vivo degradation of patellar cartilage, proteoglycans were prelabeled with $Na_2{}^{35}SO_4$. [^{35}S]Sulphate (1.85-3.7 MBq) was injected intraperitoneally 24 hours before intra-articular injection of interleukin 1, and patellae were isolated at various time points thereafter. Before intra-articular injection (t=0) one group of six mice was killed and the ^{35}S content was measured. Breakdown of proteoglycans was expressed as the loss of [^{35}S]sulphate compared with the ^{35}S content of control patellae taken at t=0.

SYNTHESIS OF PATELLAR PROTEOGLYCANS

Proteoglycan synthesis was measured ex vivo. Patellae were dissected, leaving a minimal area of non-cartilaginous tissue surrounding the cartilage. The patellae (five to six specimens) were placed in 2 ml of incubation medium consisting of RPMI-HEPES (*N*-2-hydrxyethylpiperazine-*N'*-2-ethanesulphonic acid; Flow Laboratories, Irvine, Scotland) with added

penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mmol/l) and 74 kBq $^{35}SO_4$. Labelling was continued for three hours. At the end of the incubation period the patellae were fixed overnight in 10% formalin and decalcified in formic acid (5%) for four hours. The patella could then easily be punched out of the adjacent tissue and was dissolved in 0.5 ml Luma solve (Hicol, Oud-Beijerland, The Netherlands) at 60°C for four hours. The ^{35}S-content of each patella, which is a reliable measure of the [^{35}S]glycosaminoglycan content,[43] was measured by liquid scintillation.

IODINATION OF INTERLEUKIN 1

About 25 μg interleukin 1α was radiolabelled with ^{125}I (AmershamInternational, Buckinghamshire, UK) by the Bolton and Hunter method. The iodinated protein was smaller than 17 kilodaltons as shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and more than 90% of the radiolabelled preparation could be precipitated with goat anti-mouse interleukin 1α antisera. The labelled preparation also retained full biological activity.

INTERLEUKIN 1 RETENTION MEASUREMENTS

^{125}I labelled interleukin 1 (800 units) was injected into the right knee joint and saline into the left knee joint. At various hours thereafter the ^{125}I radioactivity of both knees was measured by external gamma counting. Values for the right knee were corrected according to the value for the left knee, which represents background activity. Retention was expressed as a percentage of the initial count rate measured immediately after [^{125}I]interleukin 1 injection. In addition, quantitative measurements were made on dissected patellae.

AUTORADIOGRAPHY

Dry deparaffinised tissue sections of whole patellae were covered by a photographic K5 emulsion (Ilford, Basildon, Essex, UK), and exposed for three to five weeks. After this period the slides were developed and stained with haematoxylin and eosin.

HISTOLOGY OF KNEE JOINTS

Histological scoring of the inflamed knees was carried out after dissection and processing.[44] Standard frontal sections (6 μm) were prepared and stained with haematoxylin and eosin, Giemsa stain, or with safranin O, which enables identification of neutrophils and eosinophils. Eosinophils were not seen after interleukin 1 injection. The arthritic score was determined by grading the infiltrate and the exudate from 0 to +++, where 0=no change, and +++=large numbers of cells. The scoring of cartilage depletion was done on safranin O sections and depletion was graded from 0 to +++ in accordance with the degree of loss of staining in the superficial, metabolically active cartilage layer.

Results

SUPPRESSION OF THE PROTEOGLYCAN SYNTHESIS

Interleukin 1 induced marked changes in the metabolism of cartilage in vivo. The suppression of proteoglycan synthesis in patellar cartilage 24 hours after intra-articular injection was dependent on dose (fig 1). There was considerable inhibition of proteoglycan synthesis at low doses (3 U) of interleukin 1, and 30 U interleukin 1 (about 1 ng) was sufficient to decrease the proteoglycan synthesis to 60% of normal. Recombinant murine interleukin 1ß was as potent as interleukin 1α in the suppression of proteoglycan synthesis (table 1). The in vivo potency of interleukin 1 was further shown by the rapid appearance of the interleukin 1 effect. With 30 U of interleukin 1 the proteoglycan synthesis showed maximal suppression at 24 hours, which remained high for up to 48 hours and declined thereafter (fig 2).

When a high dose of interleukin 1 (300 U) was used the suppression was greater at 48 hours than with lower doses, though not reaching statistical significance compared with 24 hours; suppression was still observed at 72 hours. Restoration of proteoglycan synthesis was inconsistent. The mean value (75% of normal proteoglycan synthesis) was an average that included animals which showed full recovery and some animals still with full suppression. Although a lower dose of interleukin 1 (30 U) induced the same suppression at 24 and 48 hours as 300 U, there was complete recovery of proteoglycan synthesis at 72 hours and sometimes enhanced proteoglycan synthesis. An overshoot to enhanced synthesis was more marked at later time points (data not shown), and this phenomenon contributes to accelerated recovery of the depleted matrix.

Table 1: Comparison of effects of interleukin 1α (IL1α) and interleukin 1β (IL1β) on patellar cartilage homeostasis.

Dose (U)	*Synthesis at t=24 h*				*Breakdown and loss of [^{35}S]sulphate#*			
	cpm/patella		*% Of saline*		*t=24 h*		*t=48 h*	
	IL1α	*IL1β*	*IL1α*	*IL1β*	*IL1α*	*IL1β*	*IL1α*	*IL1β*
0	626 (41)	616 (76)	100	100	11	15	21	30
3	470 (95)*	555 (75)	75	90	-	-	-	-
30	290 (22)**	269 (18)**	47	44	-	-	-	-
300	242 (20)**	270 (25)*	38	43	27	22	31	37

The proteoglycan synthesis after interleukin 1 injection was significantly suppressed- * $p<0.05$, ** $p<0.001$ -as calculated with Student's t test. This representative experiment was carried out with groups consisting of six mice each. Values are mean (SD).

The ^{35}S content was determined at 24 and 48 hours and the loss of label was expressed as a percentage of the ^{35}S content at t=0 (151 (25) cpm/patella).

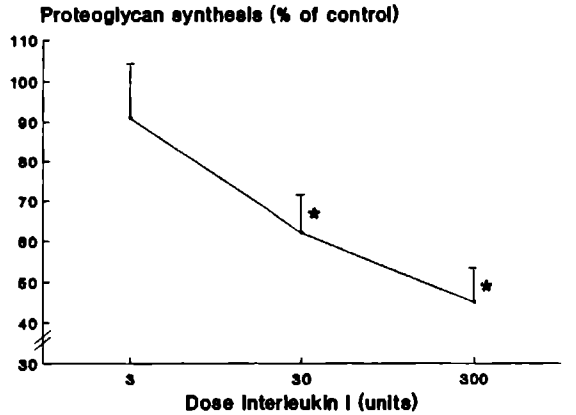

Figure 1: Proteoglycan synthesis suppression by interleukin 1.
*Interleukin 1α was intra-articularly injected into mice; the contralateral knee joint received saline. After 24 hours the patellae were dissected and cultured in tissue medium supplemented with [^{35}S]sulphate for three hours. Proteoglycan synthesis was expressed as a percentage of control ^{35}S incorporation in the contralateral. The values represents the mean (SD) of at least four experiments consisting of six animals each. Significant differences with respect to saline are indicated with * ($p<0.001$, Student's t test) and calculated from the cpm values.*

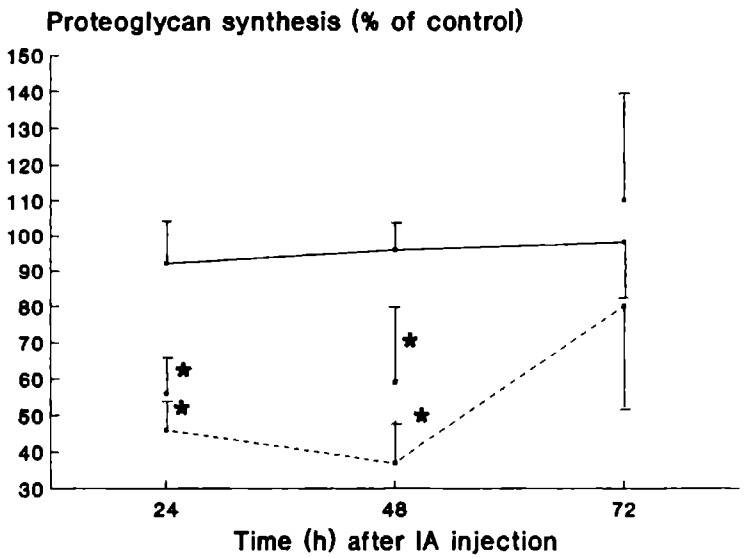

*Figure 2: Time course of proteoglycan synthesis suppression by interleukin 1. Proteoglycan synthesis was measured by [^{35}S]sulphate incorporation (see fig 1) at various days after (————) 3 units, (- - -) 30 units or (—— —— ——) 300 units of interleukin 1α injection. Each value represents the mean of at least three experiments consisting of six animals. Significant differences with respect to saline are indicated with * ($p<0.001$, student's t test).*

ACCELERATED BREAKDOWN OF PATELLAR CARTILAGE

Coincident with significant suppression of proteoglycan synthesis, interleukin 1 induced an accelerated breakdown of prelabelled proteoglycans (fig 3). A low dose of interleukin 1α (30 U) was almost as effective as the higher dose (300 U). This sensitivity of patellar cartilage to interleukin 1 had already been seen in the suppression of the proteoglycan synthesis. The basal proteoglycan breakdown rate in control patellae was 10.7 (SD 6.0)% (n=9) as measured by loss of [^{35}S]sulphate between 0 and 24 hours. In the presence of interleukin 1α (300 U) the breakdown was 23.0 (9.1)% (n=6) during the first 24 hours after injection. This means that interleukin 1 accelerated the proteoglycan breakdown rate twofold. Further accelerated loss of proteoglycans between 24 and 48 hours after interleukin 1 injection was limited. Interleukin 1ß also induced significant breakdown, but seemed less potent than interleukin 1α in a comparative experiment (table 1).

Histologically, in sections of whole knee joints, no clear cut loss of cartilage matrix could be seen 24 hours after a single interleukin 1 injection (figure 4).

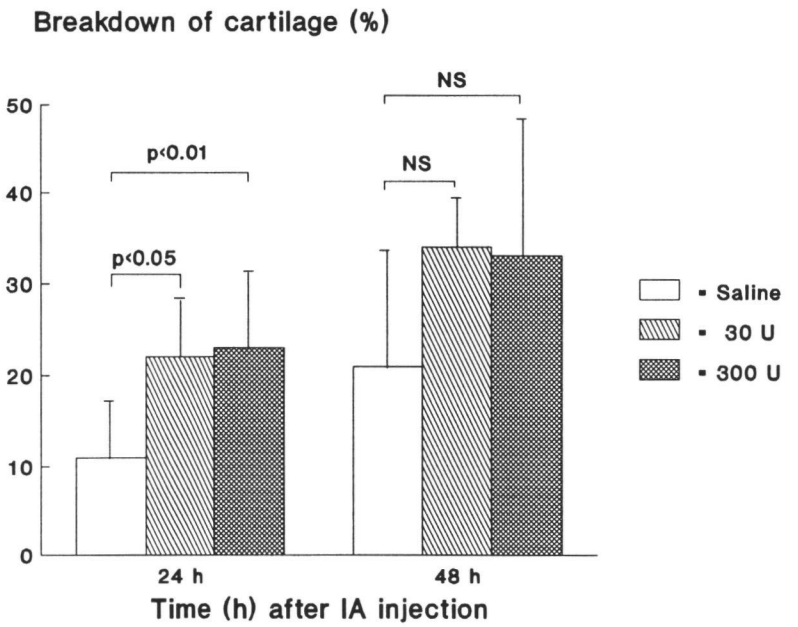

Figure 3: Enhanced breakdown of patellar proteoglycan by interleukin 1α. Animals recieved 1.85 MBq [$_3$^{5}S]sulphate intraperitoneally 24 hours before intra-articular interleukin 1 administration. The ^{35}S loss from patellae at 24 and 48 hours was expressed as a percentage of the ^{35}S-content at t=0 (235 (50)cpm). The bar of 30 units interleukin 1 represents the mean of three experiments and the 300 unit bars of 5 experiments of six animals each.

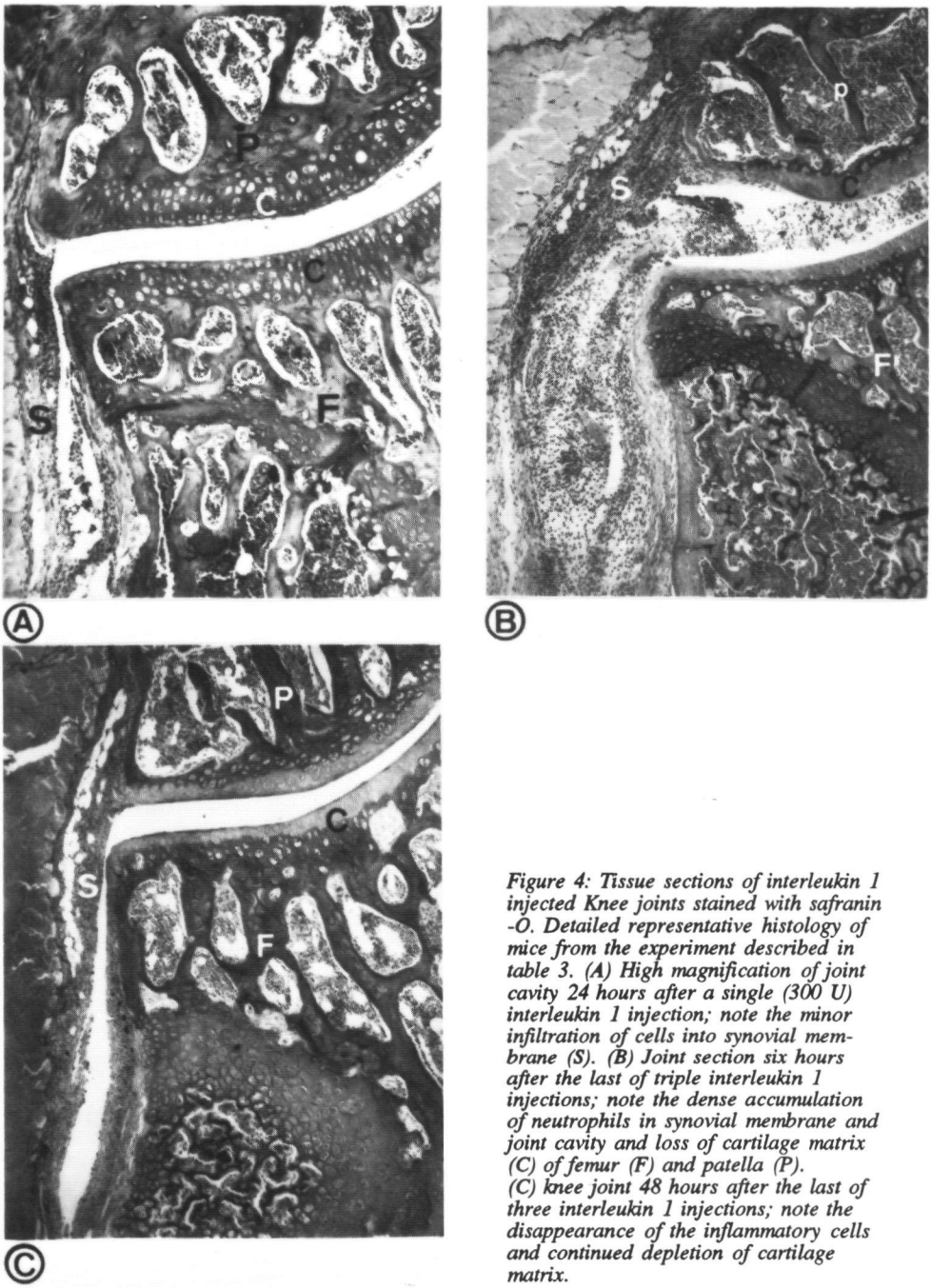

Figure 4: Tissue sections of interleukin 1 injected Knee joints stained with safranin -O. Detailed representative histology of mice from the experiment described in table 3. (A) High magnification of joint cavity 24 hours after a single (300 U) interleukin 1 injection; note the minor infiltration of cells into synovial membrane (S). (B) Joint section six hours after the last of triple interleukin 1 injections; note the dense accumulation of neutrophils in synovial membrane and joint cavity and loss of cartilage matrix (C) of femur (F) and patella (P). (C) knee joint 48 hours after the last of three interleukin 1 injections; note the disappearance of the inflammatory cells and continued depletion of cartilage matrix.

SUPPRESSION OF PROTEOGLYCAN SYNTHESIS BY INTERLEUKIN 1 AND THE EFFECT OF INFLAMMATION

To investigate whether the effect of interleukin 1 on the articular cartilage was related to its potential to induce joint inflammation we compared the effect of various doses of the arthritogen zymosan and recombinant interleukin 1. A high dose of zymosan (60 μg) caused marked suppression of proteoglycan synthesis (46%) 24 hours after intra-articular injection, and technetium uptake measurements, reflecting joint inflammation, were highly raised. When the dose of zymosan was lowered, less severe inflammation and concomitantly less suppression of proteoglycan synthesis was found, indicating that in this dose range there was a correlation between inflammation and inhibition of proteoglycan synthesis. A dose of 1.8 μg zymosan still induced significant joint swelling (Tc uptake) but the effect on proteoglycan synthesis was no longer seen. In contrast, interleukin 1α in the dose range studied did not induce enhanced technetium uptake, but there was a marked inhibition of proteoglycan synthesis (table 2).

Table 2: Comparison of the effect of zymosan and interleukin 1α on joint inflammation and proteoglycan synthesis. Values are given as means (SD).

Substance	*Dose (intra-articular)*	*Inflammation*	*Proteoglycan synthesis[ƒ]*	
		^{99m}Tc-ratio[#]	*cpm/patella*	*% Of control*
Saline	-	1.09 (0.08)	1303 (245)	109
Zymosan (μg/knee)	1.8	1.23 (0.08)	1087 (106)	99
	6.0	1.32 (0.09)*	940 (109)	76
	18.0	1.41 (O.10)*	845 (99)*	62
	60.0	1.80 (0.07)*	675 (135)*	54
Interleukin 1α	30	1.04 (0.05)	732 (51)*	64
(U/knee)	300	1.02 (0.05)	401 (31)*	35

Joint swelling was detected by enhanced ^{99m} Tc pertechnetate uptake as measured by external gamma counting. This was expressed as a ratio of the technetium uptake in the inflamed knee over that in the contralateral knee within the same animal.

ƒ Patellae were dissected and the proteoglycan synthesis was determined by [^{35}S]sulphate incorporation. In the untreated contralateral patellae the ^{35}S incorporation was stated as 100%.

Significant differences- * $p<0.001$ -with respect to the contralateral knee were calculated with the Student's t test.

Histology showed that after 24 hours there was only minor infiltration of polymorphonuclear leucocytes into the synovial membrane (fig 4).

In conclusion, the inhibition of proteoglycan synthesis by interleukin 1 must be due to a direct effect on cartilage and was not secundary to inflammation induced by interleukin 1.

INTERLEUKIN 1α RETENTION IN MURINE KNEE JOINT

The iodinated interleukin 1 (Bolton and Hunter) retained its full biological activity and molecular character. There was a poor retention of [^{125}I]interleukin 1 in the naive knee joint (fig 5). One hour after [^{125}I]interleukin 1 injection 30% of interleukin 1 was retained, and at six hours retention fell below 10%. The penetration of interleukin 1 into the patella was poor. Autoradiography carried out shortly after injection showed no distinct labelling of the cartilage compared with the joint cavity. At six hours after [^{125}I]interleukin 1 injection only 0.02 U [^{125}I]interleukin 1 was retrieved in the isolated patella and autoradiography showed that interleukin 1 was faintly dispersed in the patellar cartilage matrix. Distinct association with chondrocytes was never found.

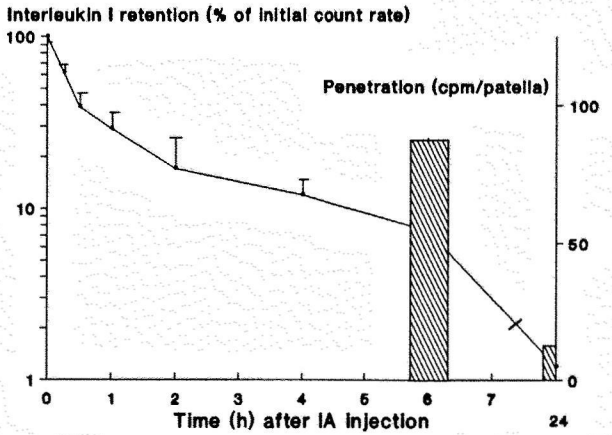

Figure 5: Interleukin 1α retention in murine knee joint and penetration into patellar cartilage. Interleukin 1 iodinated by the method described by Bolton and Hunter remained biologically active as based on costimulation of thymocyte proliferation. In naive kneejoints 800 units of [^{125}I]interleukin 1 (about 20ng or 3x10^6 cpm) was injected. The retention of [^{125}I]interleukin 1 in the knee joint was measured by external gamma counting. [^{125}I]interleukin 1 content in dissected patellae was an estimation of the interleukin 1 penetration into cartilage. Six hours after injection 80 cpm (about 0.02 units interleukin 1) could be retrieved. The proteoglycan synthesis in patellar cartilage 24 hours after injection of [^{125}I]interleukin 1 was suppressed (41%).

JOINT INFLAMMATION AND MATRIX DEPLETION AFTER REPEATED INTERLEUKIN 1 INJECTIONS

Although a single interleukin 1 (300 U) injection did not result in significant joint swelling, interleukin 1α did cause minor inflammatory changes, like scant polymorphonuclear leucocyte influx in the synovial membranes and the joint cavity at six hours with a decline in cell influx at 24 hours. When interleukin 1 was repeatedly injected every two days, however, clear cut joint inflammation was measurable at six hours (Tc uptake values in table 3).

Histologic observations showed major infiltration of polymorphonuclear leucocytes into the synovium and an exudate in the joint cavity, which persisted for up to 24 hours after the last injection and slowly declined thereafter.

Apart from infiltration of inflammatory cells, repeated injections of interleukin 1 resulted in impressive proteoglycan depletion in the articular cartilage surface layers (table 3).

Table 3: The inflammatory response of single and repeated interleukin 1α injections and the effects on cartilage matrix.

Variable	*Single IL1[injection*			*Triple IL1 injections*		
	6 h	*24 h*	*48 h*	*6 h*	*24 h*	*48 h*
Infiltration	ND	+	±	+++	++	±
Exudation	ND	±	0	+	±	0
Proteoglycan depletion	ND	0	0	++	+++	+++
^{99m}Tc ratio (mean(SD))	1.03 (0.09)	1.02 (0.05)	ND	1.22 (0.06)*	1.03 (0.06)	ND

Representative experiment in which single and triple (three times on alternate days) interleukin 1 injections were compared for their inflammatory effect, six to 48 hours after the last injection. The joint sections were scored on: infiltration of leucocytes into the synovial membrane, exudation of polymorphonuclear cells into the joint cavity, and proteoglycan depletion of cartilage matrix. Histological changes were arbitrarily represented as 0=no changes, ±=slight, +=moderate, ++=marked, +++=strong. Joint swelling was expressed by an enhanced ratio of ^{99m} Tc pertechnetate uptake, and significant differences- * $p<0.001$-were calculated by Student's t test. Repeated saline injections did not cause an enhanced ^{99m}Tc ratio. [IL1=interleukin 1.

This depletion was even enhanced at 48 hours after the last injection. It might be the result of both the accelerated cumulative breakdown (18%, table 4) and the insufficient resynthesis of proteoglycan (persistent inhibition, table 4). Although the cartilage matrix was heavily depleted of proteoglycans, the chondrocytes were still alive. No other dramatic histological disturbances in the articular components, such as bony changes, synovium haemorrhage, or cartilage cracks and loss of structure, were seen (fig 4). Proteoglycans were fully restored to the matrices seven days after withholding interleukin 1, indicating that the interleukin 1 effects were not related to mere toxicity. Repeated interleukin 1 injections into the knee joint prolonged the interleukin 1 induced disturbance of the cartilage metabolism, suggesting that prolonged exposure to interleukin 1 during chronic joint inflammation may ultimately be responsible for the cartilage damage so typical of some forms of chronic arthritis.

Table 4: Effects of single and repeated interleukin 1α injections on patellar proteoglycan metabolism.

Variable	*Substance*	*cpm/patella*[1]			*% Of control*		
		0 h	*24 h*	*120 h*	*0 h*	*24 h*	*120 h*
Breakdown		380 (156)			100		
	Saline		339 (82)	240 (44)		89	63
	Interleukin 1		311 (92)	170 (45)		82	45
Synthesis		ND			-		
	Saline		1195 (101)	1062 (145)		100	100
	Interleukin 1		421 (35)*	563 (78)*		36	53

Representative experiment in which the effect on proteoglycan metabolism was compared at 24 hours after a single injection or triple injections (120 h) of saline or interleukin 1 (300 U). Breakdown of patellar cartilage was measured as loss of [^{35}S]sulphate and expressed relative to the ^{35}S content at t=0. Proteoglycan synthesis of patellae was measured as the amount of [^{35}S]sulphate incorporated. Significant differences- * $p<0.001$ -with respect to saline were calculated by Student's t test. [Values are means (SD).

Discussion

Our observations on the effects of recombinant interleukin 1 show two distinct alterations in synovial joints: (a) induction of granulocyte accumulation in the joint and (b) disturbance of the cartilage metabolism.

A single injection of interleukin 1 was only slightly inflammatory: it induced no enhanced plasma extravasation (tables 2 and 3) at 24 hours and only minor infiltration in the synovial membrane and exudation into the joint cavity, predominantly polymorphonuclear leucocytes (table 3, fig 4). It is known that interleukin 1 stimulates vascular endothelial cells to express a neutrophil CD18 antigen. This antigen facilitated the adhesiveness of neutrophils.[13 45] Although new protein synthesis was required for this, the accumulation of neutrophils peaked at four hours but had subsided by 24 hours.[13 15] Even when we injected high doses of interleukin 1 (up to 800 U) still only minor inflammation could be observed at four and six hours (data not shown). Signs resembling experimental arthritis, like synovitis and severe cartilage matrix depletion, were only seen after repeated interleukin 1 injections; these signs were probably related to rapid interleukin 1 clearance from the joint (fig 5). The massive infiltration and major exudation of leucocytes after triple interleukin 1 injections were not a mere summation of the effects of each interleukin 1 injection. A local hypersensitivity for interleukin 1 as seen in the interleukin 1 induced local Shwartzman reaction in rabbit skin might be postulated.[46 47] Similar interleukin 1 hyperreactivity was also seen in arthritic flares of joints previously injured with bacterial cell

wall preparations,[22] and we have recently shown that interleukin 1 causes flares of smouldering zymosan or antigen induced arthritis in mice.[48] In addition, the onset of type II collagen induced arthritis in mice was enhanced by continuous administration of interleukin 1.[49]

The question arises whether inflammation was at least partially responsible for interleukin 1 induced cartilage depletion. We have shown that suppression of the proteoglycan synthesis induced by one single interleukin 1 injection was not caused by continuing inflammation. Compared with zymosan, interleukin 1 induced slight inflammation, too little to promote strong inhibition of proteoglycan synthesis (table 2). The suppression of proteoglycan synthesis after repeated interleukin 1 injections did not significantly differ from that after a single injection. Although significant inflammation was found after repeated interleukin 1 injections, it seems unlikely that the cumulative loss of [^{35}S]proteoglycan was related to this. The overall loss was 18% (table 4) and might well be explained by triple interleukin 1 insults, each resulting in 7-8% [^{35}S]proteoglycan degradation. Recent studies from Pettipher *et al* also provided evidence indicating a minor role of the inflammatory exudate in proteoglycan degradation.[21] Proteoglycan loss from cartilage induced by interleukin 1 was unimpaired in neutropenic animals,[41] and pronounced proteoglycan degradation was still found during antigen induced arthritis in neutropenic rabbits. [41] Earlier studies in inflamed air pouches already pointed to the incompetence of an acute inflammatory exudate in cartilage breakdown.[50] It may even be postulated that neutrophils prevent interleukin 1 effects as they contain a specific interleukin 1 inhibitor.[51 52]

The marked depletion of cartilage matrix after repeated interleukin 1 injections as shown by histology (fig 4) is probably the final result of both cumulative breakdown of proteoglycan (18%, table 4) and prolonged suppression of proteoglycan synthesis (50%, table 4). At day 1 after a single interleukin 1 injection about 10% proteoglycan depletion was found, and the lack of clear-cut proteoglycan loss on histologic sections taken at that time (fig 4) indicates that this degree of depletion is beyond the detection limit. The marked depletion after repeated interleukin 1 injections must therefore reflect a much higher degree of proteoglycan depletion and this may only be explained by a considerable lack of sufficient resynthesis of new proteoglycan. Kinetic studies on this subject are in progress. Earlier studies with purified interleukin 1 preparations given once a day yielded high degrees of proteoglycan depletion,[39 40] but the possibility that this was caused by other mediators present in the interleukin 1 preparation cannot be excluded. Moreover, in those studies the recovery was also very poor during the days after interleukin 1 exposure,[39 40] in sharp contrast with the full recovery of the matrix seen with recombinant interleukin 1 in rabbits[41] and in mice in this study. Five days after the last of triple interleukin 1 injections inflammation was absent and cartilage was again heavily stainable with safranin-O. At that stage an overshoot to enhanced proteoglycan synthesis was evident (data not shown).

The most outstanding action of interleukin 1 in vivo was its impressive longlasting inhibition of proteoglycan synthesis. This inhibition was not related to toxicity as full recovery was seen later. As stated above this inhibition contributes to proteoglycan depletion in the metabolically active murine cartilage matrix. It remains to be seen whether the inhibition of proteoglycan synthesis contributes in a similar degree to matrix depletion in older animals or in other species as it is obvious that it depends on turnover rates. Preliminary studies in our laboratory show that interleukin 1 induced even more prolonged inhibition of proteoglycan synthesis in the cartilage of old mice (18 months).

A recent study with isolated chondrocytes showed the onset of interleukin 1 induced suppression of proteoglycan synthesis six hours after first exposure and complete recovery to normal synthesis was found 48 hours after withholding interleukin 1,[53] effects in agreement with our in vivo results.

We found a comparable degree of suppression of proteoglycan synthesis and breakdown with recombinant interleukin 1α and ß (table 2); this is consistent with other studies. The effects of interleukin 1 on rabbit chondrocytes (prostaglandin E_2 synthesis, phospolipase A_2 secretion, neutral protease release) or on human chondrocytes (caseinase secretion, prostaglandinE_2 production) or on cartilage resorption in explants show that both interleukin 1 species are almost equally potent.[54-57] This is not surprising because chondrocytes posses a common class of receptors for interleukin 1α and ß, with similar affinity.[58 59] The overall effect of interleukin 1 on cartilage was dramatic: a joint suppression of proteoglycan synthesis and enhancement of proteoglycan breakdown, which amplifies matrix depletion. Both phenomena are clearly seen in experimental arthritis models and interleukin 1 seems a likely cause of both effects. The follow up of interleukin 1 studies may elucidate the exact role of interleukin 1 in various forms of arthritis.

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CHAPTER 4

PROTEOGLYCAN LOSS AND SUBSEQUENT REPLENISHMENT IN ARTICULAR CARTILAGE AFTER A MILD ARTHRITIC INSULT BY IL-1 IN MICE: IMPAIRED PROTEOGLYCAN TURNOVER IN THE RECOVERY PHASE

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Proteoglycan loss and subsequent replenishment in articular cartilage after a mild arthritic insult by IL-1 in mice: Impaired proteoglycan turnover in the recovery phase

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Abstract. The reparative responses of articular cartilage after an arthritic insult have not been studied extensively to this day In the present study, we injected interleukin-1 (IL-1) into knee joints of mice to provoke a mild and transient arthritic insult, and characterized both the catabolic and the subsequent recovery phase In the catabolic phase, which lasted 2 days after IL-1 injection, proteoglycan (PG) breakdown was profoundly accelerated and PG synthesis was markedly inhibited Sulfation and polysaccharide synthesis were not affected, yet the number of chondroitin sulfate chains was decreased The general chondrocyte protein synthesis was not inhibited by IL-1 IL-1 injected every other day for a total of three injections prolonged this catabolic phase and resulted in frank loss of articular cartilage proteoglycans In the recovery phase, started 3 days after IL-1, PG synthesis was enhanced (1 7 times the normal) and proteoglycans had normal hydrodynamic properties Remarkably, PG degradation was significantly decreased (approximately 50% of the normal) Zymographic analysis demonstrated enhanced expression of gelatinolytic activities in the extracts of the articular tissues shortly after IL-1 exposure and decreased levels in the recovery phase We found that the overshoot of PG synthesis and impaired degradation act together to facilitate full cartilage repair 7 days after the last of the three IL-1 injections

Key words: Patella – Chondrocyte metabolism – Gelatinase-activities – Zymography – Glycosaminoglycan – Interleukin-1

Cartilage, a highly specialized coating tissue on bones, forms the articular joint surfaces The macromolecular structure of this tissue consists of a collagen type II fibre frame embedded in proteoglycans The proteoglycans are large aggregates (aggrecan) of highly sulfated polysaccharides and they create a large osmotic swelling pressure [1] Water uptake will tense the collagen network creating a resilient structure which enables smooth joint movements and can restrain compressive loading stress [2] In contrast to collagen, the proteoglycans are continuously turned over and chondrocytes maintain their extracellular matrix by synthesis of proteoglycans The chondrocyte metabolic activity is controlled by a range of cytokines and growth factors [3] Cartilage PG loss can occur after a trauma, immobilization of the joint, or as a consequence of a local inflammation

In mice, we demonstrated that an intra-articular injection of the inflammatory cytokine, interleukin-1α, induced suppression of PG synthesis and a concomitant accelerated breakdown [4, 5] Furthermore, we recently elucidated the role of IL-1 in the antigen-induced arthritis (AIA) and collagen-induced arthritis in mice Suppression of chondrocyte PG synthesis in AIA could be prevented by treatment with neutralizing anti-IL-1 antibodies [6, 7], implicating a key role of IL-1 in this process

At present, the bulk of research is focused on unraveling the mechanism behind cartilage depletion and on cessation by enzyme inhibitors and cytokine antagonists [8] Due to low cellularity, the capacity of cartilage to repair major PG loss is limited, but not refractory The ensuing replenishment of proteoglycans is a neglected area.

In vivo reparative responses of cartilage have been examined after papain digestion of the matrix, immobilization of the joint, and after traumatizing by full-tickness cartilage defects or transection of the anterior cruciate ligament The last mentioned is also a model of osteoarthritis (OA) in which osteophytes stabilize the joint followed by hypertrophic cartilage repair (fibrocartilage) [9]

In the present study, IL-1 action on the murine joint was examined by detailed analysis of cartilage PG metabolism, and metalloproteinases expression in tissues from the *capsula articularis* and patellae In addition, the IL-1-induced cartilage insult was utilized as an *in vivo* model system to characterize further the subsequent recovery phase

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Materials and methods

Animals and induction of an arthritic insult

The experiments were performed on male C57Bl/6 mice aged 8–10 weeks. Mice were obtained from our Central Animal Laboratory breeding facility and were free of specific pathogenic viruses. They were fed a standard diet and tapwater *ad libitum*. Mice were injected intra-articularly along the suprapatellar ligament into joint cavity. The right knee joints received IL-1α (10 ng) and the contralateral knee joint received an equivalent volume (6 µl) of saline and served as a within animal control. We demonstrated previously that saline or 3 U (0·1 ng) of IL-1 had no significant effect on chondrocyte metabolism [4]. Murine recombinant IL-1α cloned in Escherichia coli [10] had less than 1 ppm endotoxin in the Limulus lysate assay. Bioactivity was checked in the one stage bioassay a coculture of the IL-1-specific subclone of the murine thymoma cell line EL-4 designated NOB-1 which produces IL-2 and IL-4 together with the IL-2-responsive CTLL2 cells. Half-maximal proliferation was obtained at a concentration of 1 pg/ml of IL-1 and could be blocked by rabbit anti-IL-1 antibodies [6].

Analytical procedures

Assessment of proteoglycan synthesis. Patellae with a minimal amount of adjoining soft tissue (6 pieces) were incubated for 3 h in 2 ml RPMI 1640 (Dutch modification, Flow Laboratories, Irvine, Scotland) supplemented with gentamycin (50 µg/ml), L-glutamine (2 m*M*) and 40 µCi ^{35}S-sulfate (Du Pont de Nemours, s Hertogenbosch, The Netherlands) at 37 C in a humidified 5% CO_2 atmosphere. Thereafter patellae were thoroughly washed, fixed in 10% formalin and subsequently decalcified in 5% formic acid. Patellae were punched out of the adjacent tissues and dissolved in 0·5 ml Lumasolve (Hicol Oud Beijerland, The Netherlands) at 60 C. The ^{35}S-content of each patella was measured by liquid scintillation counting (LSC). More than 95% of the radiolabel was incorporated into the proteoglycans by this method [11].

Assessment of proteoglycan breakdown. Mice were injected with 50–100 µCi ^{35}S sulfate intraperitoneally. After 24 h and at later timepoints (indicated in the legends) patellae were dissected and processed as described above. Breakdown was either expressed as a ratio of ^{35}S-content of treated over untreated contralateral joints or expressed as a percentage of the first timepoint (24 h) taken, as will be indicated in the corresponding experiments.

Glycosaminoglycan measurements of patellar cartilage. Estimation of sulfated glycosaminoglycan (GAG) content in cartilage was carried out using the colorimetric method of Farndale [12]. After dissection, patellae were fixed in ethyl alcohol (96%) overnight and decalcified in 5% formic acid for 4 h before use. The cartilage layer was stripped from the patella with a pair of tweezers and subsequently digested with 200 µl of 5 mg/ml papain (Type IV, Sigma, St. Louis, MO) in 0·1 *M* sodium acetate, pH 6·5, 10 m*M* L-cysteine and 50 m*M* di-sodium EDTA per patella overnight at 60 C. The GAG content per patella was estimated by the dimethylmethylene blue dye binding at 535 nm using shark chondroitin sulfate (Sigma) as a standard.

Measurements of hyaluronic acid synthesis. Patellae ($n=6$) were incubated for 20 h in 2 ml RPMI tissue-culture medium supplemented with 100 µCi ^{3}H-glucosamine and 30% normal mouse serum to maintain proteoglycan synthesis at the *in vivo* level. Supernatants of a papain matrix digest (see above) were applied to a cellulose acetate strip. Hyaluronic acid and chondroitin sulfate were separated by electrophoresis (60–10 mA, 80 V). The spots were visualized by 0·1% alcian blue in 5% acetic acid and 10% ethyl alcohol, cut out and the amount of ^{3}H-label was measured in aqualuma by LSC. As a specificity control supernatants were dialyzed and vacuum dried and treated with 100 U of hyaluronidase (*Streptomyces*) in 0·1 *M* sodium acetate 0·15 *M* NaCl pH 4·0. After 20 h incubation at 37 C, the supernatant was applied to the cellulose strips.

Gel permeation chromatography of proteoglycans. Since bovine proteoglycan monomers are included more on Sephacryl S-1000 (Kav 0·38) than on Sepharose CL-2B (Kav 0·28) fractionation on Sephacryl S-1000 allowed a more clear resolution [13] and a distinct separation of proteoglycan monomers with large or small hydrodynamic volume of murine patellar cartilage [14]. Patellae were cultured for 4 h in RPMI 1640 DM medium containing ^{35}S-sulfate (10 patellae per 40 µCi/ml) followed by thorough rinsing in physiological saline. Proteoglycans were extracted immediately thereafter or the labelling interval was followed by a 48 h chase period in RPMI supplemented with hrIGF-1 (0·5 µg/ml serum equivalence). Subsequently the patellae were decalcified in 3·5% Na_2EDTA solution (osmolarity 270 mmol/kg) (Sigma Chemical Company, St. Louis, MO) for 4 h and the cartilage layer was stripped from the underlying bone using forceps. The cartilage layers were ground with an all-glass mortar and pestle followed by proteoglycan extraction at 4 C for 48 h in 4 *M* guanidine HCl (GuHCl) in 0·5 *M* sodium acetate buffer (pH 5·6) in the presence of protease inhibitors (0·01 *M* EDTA, 0·1 *M* 6-aminocaproic acid (Sigma), 0·005 *M* benzamidine hydrochloride (Sigma), 5 mg/ml trypsin inhibitor (Sigma), 0·005 *M* iodoacetate (Sigma)). This extraction procedure resulted in the extraction of over 90% of the incorporated radiolabel. Extracted proteoglycans were supplemented with 1 mg of an unlabelled bovine proteoglycan mixture as a carrier and applied to an analytical Sephacryl S-1000 column (100 × 1·6 cm). The column was equilibrated and eluted with 4 *M* GuHCl (dissociative). The column was eluted at a flow of 9 ml/h and fractions of 3 ml were collected. Aliquots of each column fraction were analyzed for glycosaminoglycans (Farndale) and radioactivity (LSC). Slices of articular cartilage obtained from the metacarpal joints of bovine were extracted with 4 *M* GuHCl and dialyzed against 0·5 *M* GuHCl to reassociate into aggregates. To void volume (V_0) of the column was characterized by these bovine proteoglycan aggregates under associative elution conditions (0·5 *M* GuHCl) (elution position in fraction 29). Bed packing was checked with dextran blue 2000 (2×10^6 MW), and eluted with a Kav of 0·64 (dissociative running conditions). Total bed volume (V_t) was characterized by unbound ^{35}S-sulfate which eluted at fraction 67 (dissociative running conditions). All samples were analyzed on the same column and reproducibility was checked by the comparison of the elution profile of the unlabelled bovine carrier proteoglycans.

Characterization of newly synthesized chondrocyte proteins. Patellae ($n=10$ per group) were dissected with a minimal amount of surrounding tissue and incubated with 2 ml methionine-free RPMI tissue-culture medium containing 100 µCi ^{35}S methionine plus cold L-methionine to a final concentration of 0·5 mg/L, 5% FCS and 1·5 mg/L tunicamycin for 4 h at 37 C in a humidified atmosphere. Patellae were then washed thoroughly and decalcified in a physiological aqueous solution of 3·5% (w/v) Na_2EDTA, glucose (1 g/L), supplemented with minimal essential amino acids (MEM 50× amino acid solution, Whittaker M.A. Bioproducts, Walkersville, MD), 2 h at 37 C or 4 h at 4 C. The patellar cartilage could be removed from the bone using forceps. Chondrocytes were freed from the cartilage by enzymatic digestion of the cartilage matrix with 2000 U/ml collagenase IA (from *Clostridium histolyticum*), 20 U/ml pronase E (Protease type XIV from *Streptomyces griseus*) and 0·5 U/ml chondroitinase ABC (from *Proteus vulgaris*) and 2% BSA in HAM's F12 culture medium. After 45–60 min the chondrocytes were centrifuged at 250 × *g* for 10 min and resuspended in 0·1% EDTA in saline. Viability was checked by trypan-blue exclusion and the cell number was determined.

Chondrocytes were washed in PBS and spun-down at 300 × *g* for 5 min, and resuspended in 50 µl lysis buffer (50 m*M* KCl, 10 m*M* NaCl, 1 m*M* EDTA, 50 m*M* potassium phosphate, 10% NP-40 and 0·25 m*M* PMSF). Nuclei were separated in an Eppendorf centrifuge at 1200 × *g*. The supernatant was diluted 1·5 with sample buffer (0·011 g/ml dithiothreitol, 0·1 g/ml SDS in 0·4 *M* Tris HCl pH 6·8

with 45% glycerol and bromophenol blue) and heated at 100 °C for 2 min.

Gel analysis. Equal amounts of radiolabel were subjected to SDS–polyacrylamide gel electrophoresis (Daiichi 10–20% gradient gels, ISS-enprotech, Massuchusetts, USA) and run for 1 h at 40–60 mA, constant current. Gels were silver-stained according to the manufactures procedures (2-D-silver stain-II Daiichi) and afterwards submerged in an autoradiography enhancer (EN^3HANCE, Biotechnology Systems NEN, Massachusetts, USA) and vacuum dried. Gels were exposed to X-ray film for a defined length of time at −70 °C. All films were scanned using a Molecular Dynamics laser scanner and protein bands were quantitated using PDQ-Scan software (Protein Databases, Inc., USA). Protein bands were matched between lanes on the gels of each experiment, and apparent molecular weight assignments were made based on internal standards: Phosporylase β (95.5 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa), and cytochrome C (12.4 kDa).

Isolation of metalloproteases from patellae. Patellae immediately frozen in liquid nitrogen were punched out of their surrounding soft tissue (joint capsule) without defrosting, and both were immediately extracted for 48 h at 4 °C in a 10 m*M* cacodylate buffer (pH 6) containing 0.3 *M* NaCl, 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 0.02% (v/v) NaN_3, 0.1% (v/v) Triton X-100, and 0.7 mg/l pepstatin. In some experiments, cartilage layers were cut off from the underlying bone and extracted. Samples were stored at −70 °C, and before use mixed with an equal volume of the sample buffer (10% sodium dodecyl sulfate (SDS), 4% sucrose, 0.02% NaN_3) with bromophenol blue, and subjected to zymography.

Substrate–gel analysis (zymography) of metalloproteinases. Slab gels (84 × 90 × 1.0 mm) containing the appropriate gelatin substrate (2.5 mg/10 ml, Difco Lab., Detroit, MI) were copolymerized in 7.5% polyacrylamide as described [15]. Slab gels were run at 4 °C, at a constant voltage (200 V). After electrophoresis, the slab gels were washed twice for 20 min in 50 m*M* Tris (pH 7.5), 5 m*M* $CaCl_2$, 1 m*M* $ZnCl_2$, 0.02% (w/v) NaN_3, and 2.5% (v/v) Triton X-100 at room temperature to remove the SDS. To detect gelatinase activity, the gels were incubated in 50 m*M* Tris (pH 7.5), 5 m*M* $CaCl_2$, 1 m*M* $ZnCl_2$, 0.02% (w/v) NaN_3, and 1% (v/v) Triton X-100, with or without 1 m*M* APMA, for 18–48 h at 37 °C. Gels were stained in 0.25% (w/v) Coomassie blue G250 in 50% methanol, 7% acetic acid, and destained in 40% methanol, 10% acetic acid. Enzymes were detected as a clear band within the blue background. Zymography allowed us to detect both proenzymes and active enzymes based on their apparent molecular weights and in their original ratios. For this, we activated proenzymes with APMA after the electrophoretic separation and not before.

Statistical analysis. Data are expressed as mean values ± standard deviation (SD) unless stated otherwise. Statistical significance was tested using the Mann–Whitney U test.

Results

Cartilage metabolism after a single IL-1 injection, the catabolic phase

Chondrocyte protein synthesis. Murine recombinant IL-1α (10 ng) was injected intra-articularly into the murine knee joint. Twenty-four hours later, protein synthesis of patellar chondrocytes was measured by the incorporation of ^{35}S-methionine *ex vivo*. The newly synthesized proteins were retained intracellularly by blocking the glycosylation/secretion pathway with tunicamycin [16]. IL-1 did not lead to a significant inhibition (8%) of the overall protein synthesis as compared to chondrocytes in normal cartilage. Detailed analysis of these chondrocyte-derived proteins by PAGE did not reveal significant changes in preexisting proteins, whereas of the newly synthesized proteins, the expression of a 33 kDa protein became markedly increased (Fig. 1).

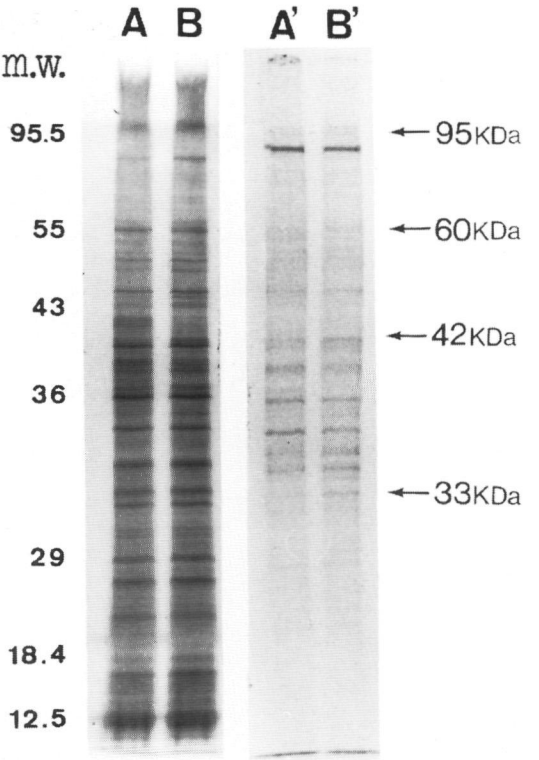

Fig. 1. Electrophoretic separation of chondrocyte intracellular proteins. The preexisting (A, B; silver-stained), and the newly synthesized proteins (A′, B′; autoradiography) were analyzed on a 10–20% polyacrylamide gel. 25.000 CPM (1–1.5 µg protein) was applied to each lane. Lane A and A′, normal chondrocytes; lane B and B′, IL-1α (10 ng)-treated cartilage (24 h). Autoradiographs were laser-scanned and apparent relative increases of matched bands between lane A′ and B′ were indicated by arrows, with their appropriate molecular weights.

Chondrocyte proteoglycan synthesis. Chondrocyte proteoglycan synthesis, as measured by ^{35}S-sulfate incorporation, was markedly suppressed after IL-1 (10 ng) exposure (Table 1). IL-1 did not cause alteration in the enzyme activities of the sulfation pathway as identical results were obtained by ^{3}H-glucosamine incorporation. Electrophoretic separation of the polysaccharide chains of the newly synthesized proteoglycans demonstrated that the amount of ^{3}H-labelled chondroitin sulfate chains was decreased, whereas the amount of ^{3}H-labelled hyaluronic

Table 1 Effect of IL-1 on the synthesis of chondroitin sulfate chains and hyaluronic acid of patellar cartilage separated by cellulose acetate electrophoresis

	Label[b]	Radioactivity[a]		
		Contralateral	IL-1 injected	IL-1/contralateral ± SD
Patella	^{35}S-sulfate	764 ± 138	400 ± 59*	0 53 + 0 12
Patella	^{3}H glucose	2485 ± 511	1603 ± 304**	0 66 ± 0 15
CS	^{3}H-glucose	2067 ± 387	1182 ± 226**	0 60 ± 0 20
HA[c]	^{3}H-glucose	207 ± 161	187 ± 105ns	0 90 ± 0 33

[a] Whole patellae extracts cellulose acetate spots of chondroitin sulfate (CS), and hyaluronic acid (HA) were counted and expressed as CPM ± SD or as a ratio
[b] Patellae (n = 6) 24 h after a single IL-1 injection and their matched contralateral untreated joints were incubated with ^{35}S-sulfate (20 μCi ml) for 3 h or ^{3}H glucosamine (glucose) (100 μCi ml) for 20 h in serum enriched medium
[c] Approximately 85% of ^{3}H glucosamine labelled HA was hyaluronidase-degradable material
p-values compared with the contralateral joints (Mann Whitney) * $p < 0.001$, ** $p < 0.05$ ns, not significant

Table 2. Breakdown of newly synthesized and the more processed proteoglycans by IL-1

Proteoglycans evaluated	Period after IL-1 challenge (days)	Ratio ^{35}S-content[a] Il -1 contralateral (± SD)	Accelerated loss of label by IL-1 (1 − ratio) × 100 (%)
New[b]	1	0 83 ± 0 05	17*
Processed[c]	1	0 82 ± 0 04	18*
New[b]	9	0 76 ± 0 11	24*

[a] Mean ratio (n = 6) from ^{35}S-incorporation of IL-1 injected over contralateral kneejoints calculated from paired measurements of six mice each Average CPM value of contralateral joints was 158 ± 28 cpm (n = 24)
[b] IL-1 effect on newly synthesized (IL-1 injected 2 days after ^{35}S-labelling) Two IL-1-challenge periods were studied 1 and 9 days over which degradation was quantitated
[c] IL-1 effect on processed proteoglycans (IL-1 injected 10 days after ^{35}S-labelling)
* $p < 0.05$ compared with the contralateral joints (Mann–Whitney) Accelerated loss by PBS was 1 ± 3%

acid chains remained the same after IL-1 exposure (Table 1) This excludes an altered glucosamine precursor pool in chondrocytes

Matrix proteoglycan breakdown Interleukin-1 injected 2 days after ^{35}S-sulfate labelling of the articular proteoglycans resulted in an accelerated breakdown of 17% within the first 24 h (Table 2) To exclude the possibility that IL-1 only induced degradation of a small subpopulation of newly synthesized proteoglycans, the effect of IL-1 on more processed proteoglycans was studied For this, the IL-1 injection was delayed for 10 days after ^{35}S-sulfate injection With 18% enhanced degradation, the more processed matured proteoglycans were as susceptible to the IL-1 induced degradation The measured cumulative loss of 24% of ^{35}S-proteoglycans 9 days after a single IL-1 injection demonstrated that the major loss occurred within the first 24 h after IL-1 injection, and that the remaining ^{35}S-labelled proteoglycans had a normal (not impaired) turnover (Table 2)

Cartilage proteoglycan depletion by repeated IL-1 injections, the catabolic phase

Proteoglycan turnover Intra-articularly injected IL-1 is rapidly cleared from the joint with a half-life of 30 min though proteoglycan synthesis suppression lasted for 2–3 successive days [4] IL-1 injected every other day for a total of three injections, inhibited proteoglycan synthesis to the same extent (≈40%) as a single IL-1 injection (Table 3) Cartilage proteoglycan breakdown was also extended after repeated IL-1 injections A cumulative accelerated breakdown of 36% was found, as compared to 13% after a single injection (Table 3)

Glycosaminoglycan content patellae The prolonged suppression of proteoglycan synthesis and a repeated evocation of degradation may have a profound impact on articular cartilage proteoglycan content The patellar cartilage matrix was dissected from the bone and digested with papain Total GAG content of patellar cartilage was determined to be 2 75 ± 0 6 μg (n = 70 patellae) in normal cartilage and was significantly reduced (20 ± 5%, mean of 4 experiments) in the 3 × IL-1-treated knee joint (Table 4) The IL-1-induced GAG-depleted cartilage was almost completely replenished within 7 days after the third IL-1 injection (Table 4)

Recovery of proteoglycan-depleted cartilage, the reparative phase

Proteoglycan synthesis Both a single and three IL-1 injections caused a marked inhibition of proteoglycan

Table 3. IL-1 effect on *in vivo* proteoglycan turnover in patellar cartilage

Number of injections[a]	PG synthesis: Ratio of IL-1/saline joint (± SD)[b]	Proteoglycan breakdown[c]		
		Remaining ^{35}S-PG's (%)		Accelerated loss by IL-1 (A − B)/A (ratio ± SD)
		Saline A	IL-1 B	
1×	0.55 ± 0.12* (n = 120)[d]	81 (n = 18)	70 (n = 18)	0.13 ± 0.07
3×	0.58 ± 0.08* (n = 48)	51 (n = 30)	33 (n = 30)	0.36 ± 0.08**

[a] IL-1α (10 ng) was injected intraarticularly once (day 1) or three times (on day 1, 3 and 5), and the contralateral joints received saline at the same time
[b] PG synthesis was measured 24 h after the single (day 2) or after the third injection (day 6)
[c] ^{35}S-sulfate was injected on day 0. On day 1, the ^{35}S-sulfate content of patellae (n = 6) was measured and the content at later timepoints was expressed as a percentage of the amount on day 1. From the absolute incorporation, the accelerated loss by IL-1 was calculated and expressed as a ratio ± SD
[d] Number of mice evaluated is stated in parentheses
Different from saline-injected joint at * $p \leq 0.001$, at ** $p < 0.05$

Table 4. Diminished glycosaminoglycan content of patellae after IL-1 treatment

Time in days[a]	Effect on PG synthesis	GAG-content in µg/patella[b]		IL-1 effect (%)
		Contralateral	IL-1 injected	
2	55%	2.47 ± 0.14	1.91 ± 0.12	− 23*
7	170%	2.75 ± 0.17	2.61 ± 0.30	− 5

[a] Indicated time of days after the last of triple IL-1 injections
[b] GAG content of the patellae was measured by the Farndale assay, and values represent the mean of four measurements consisting of two patellae each
* $p < 0.05$, compared with the contralateral joints (Mann–Whitney)
Mean GAG loss calculated from a total of four identical experiments: day 2 (20 ± 5%); day 7 (7 ± 4%).

synthesis which was followed within 2–3 days by an overshoot of PG synthesis to supranormal levels (Fig. 2). The recovery of PG synthesis was significantly faster on day 3 of three IL-1 injections than after a single IL-1 injection ($p < 0.01$). The enhancement in PG synthesis after repeated IL-1 injections peaked on days 4–5 after the last injection with a mean stimulation index of 2.31 ± 0.59 (mean of 7 experiments) compared to the contralateral (noninjected) joints. Contralateral joints were unaffected by the IL-1 regimen when compared to joints of untreated animals there was no stimulation (1.01 ± 0.16, n = 4 experiments). Two weeks later, normalization of the PG synthesis rate occurred.

Hydrodynamic properties of proteoglycans. The hydrodynamic volume of the newly synthesized proteoglycans was analyzed by gel permeation chromatography immediately after pulse labelling and after a 48 h chase. As shown in Fig. 3A, B, the newly synthesized proteoglycans in normal cartilage and the unlabelled bulk of preexisting matrix proteoglycans monomers had a K_{av} (of ≈0.44 and are considerably smaller than proteoglycan monomers from bovine cartilage (K_{av} 0.38) as described by others [17, 18], with shorter glycosaminoglycan chains [9]. Proteoglycans synthesized in the recovery phase had an identical hydrodynamic volume as untreated cartilage

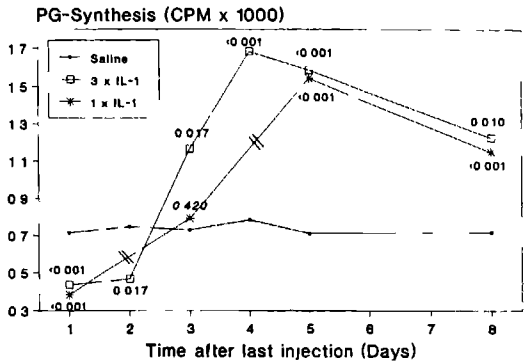

Fig. 2. Time course of patellar proteoglycan synthesis after one or three IL-1α (10 ng) injections on alternate days. Proteoglycan synthesis was measured by ^{35}S-sulfate incorporation (n = 6 per group). IL-1-treated cartilage which is different from saline-treated cartilage at p-values is indicated in the figure.

(Fig. 3C). A 48 h *in vitro* chase did not alter the hydrodynamic volume of these ^{35}S-labelled proteoglycans (Fig. 3B–D).

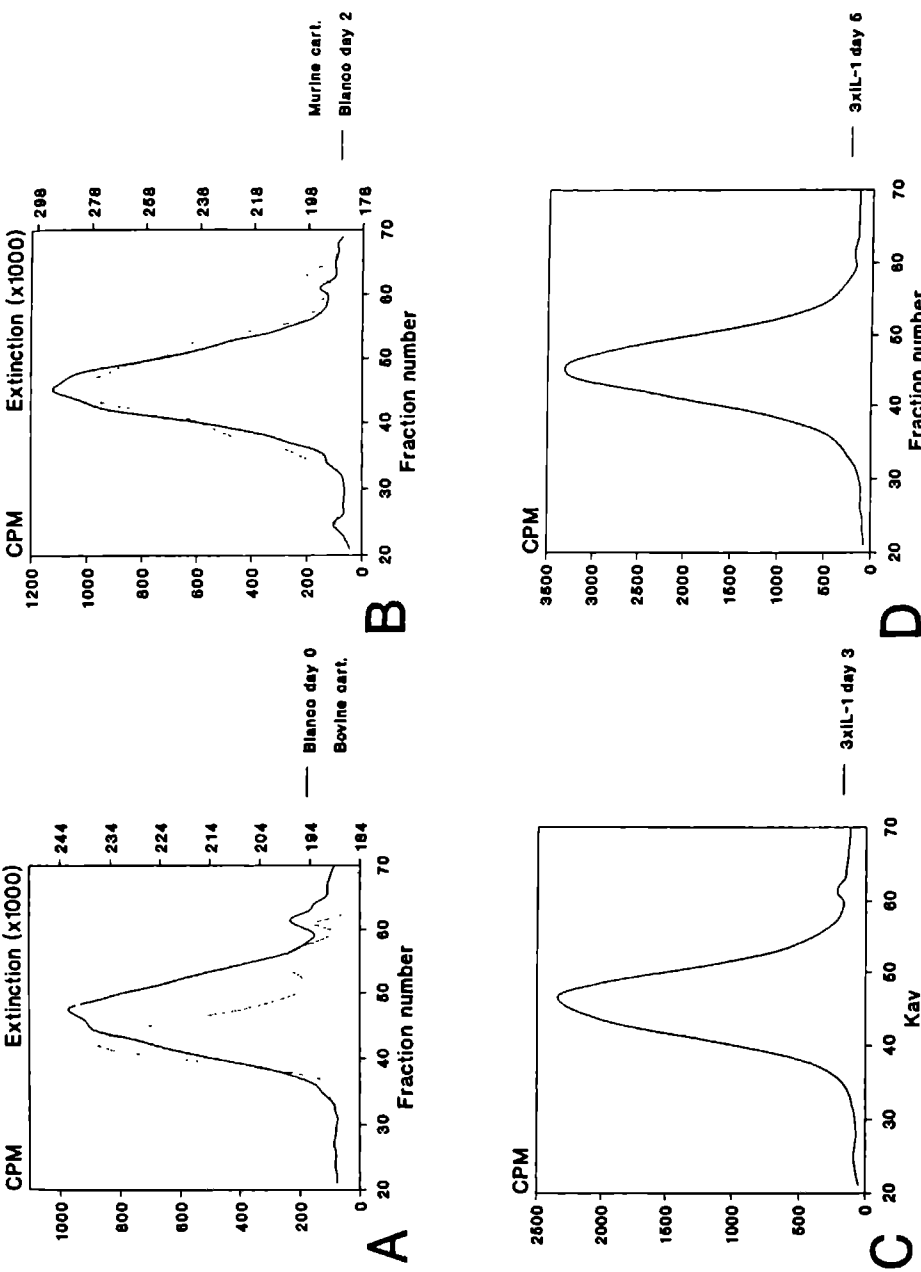

Fig. 3. Hydrodynamic volume of murine proteoglycan monomers. 4M-GuaHCl extracted patellar proteoglycan monomers were analyzed on a Sepharcryl S-1000 column. Patellae were pulse labelled with ^{35}S-sulfate and proteoglycans were extracted either directly ($n = 10$ patellae, A and C) or after an *in vitro* chase period of 48 h ($n = 20$ patellae, B and D). Solid lines represents ^{35}S-labelled proteoglycan monomers. Figs. A and B, normal-untreated patellae; Figs. C and D, patellae from day 3 after 3 × IL-1. Elution profiles of unlabelled preexisting proteoglycan monomers are indicated by the dashed lines. Fig. A, from bovine cartilage; Fig. B, from murine patellar cartilage.

Breakdown of newly synthesized proteoglycans. Degradation of proteoglycans synthesized on day 5 after the last IL-1 injection, at the time of supranormal proteoglycan synthesis, was markedly reduced: 23% compared to 60% loss over a 48 h chase period in control patellae (Table 5). This circumstantial evidence of impaired breakdown of the newly synthesized proteoglycans was further substantiated *in vivo*. Mice were injected with ^{35}S-sulfate on day 3 after the last IL-1 injection. Proteoglycan synthesis in the IL-1-treated knee was two times higher than in the normal contralateral knee joint (Table 6). Total loss of ^{35}S-proteoglycans labelled in the recovery phase was 33% after 6 days in the IL-1-treated knee and this was a significant reduction compared to the 53% loss in the contralateral normal cartilage (Table 6).

Expression of gelatinases in both phases of IL-1-treated articular tissues

The accelerated degradation of cartilage proteoglycans coincide with enhanced expression of gelatinolytic activities in both patellae and tissues of the joint capsule (Fig. 4). Extracts were prepared from the joint capsule tissue, the whole patellae and cartilage slices on days 1 and 5 after the last of triple IL-1 injections. These tissue extracts were subjected to gelatin–substrate PAGE and gelatinolytic proteases were demonstrated as clear bands in the dark-stained gel.

In the extracts of whole patellae, consistent expression of four distinct gelatinolytic bands were found: two small clearing bands of approximately 218 and 242 kDa, a moderate band of 59 kDa and a major band of 97 kDa (Fig. 4). After IL-1 exposure, markedly enhanced expression was seen and additional minor bands arose. No clearing bands emerged in gelatin gels incubated in the presence of 1 m*M* EDTA, or using irrelevant substrates, e.g., serum albumin, and fibrinogen, suggesting that the gelatinolytic bands could originate from metalloproteinase activity. When the cartilage was stripped from the patellae, the pattern was identical, although less pronounced due to the minimal amount of cartilage isolated with this procedure.

In the tissues of the joint capsule, 59 kDa appeared to be the major band. Shortly after phosphate-buffered saline (PBS) injection, the 97 kDa band was as prominent, whereas after IL-1 exposure a similar set of bands was noted as compared to the patella. In contrast, this expression returned to the baseline on day 5, whereas moderately enhanced expression was still present in the patella.

Table 5. Reduced breakdown of proteoglycans synthesized in the recovery phase during an *in vitro* chase.

Chase *in vitro*[a]	^{35}S-sulfate content	
	Contralateral (CPM ± SD)	IL-1 injected (CPM ± SD)
0 h	10166 ± 1540	18153 ± 5323
48 h	4070 ± 702* (60%)	14011 ± 1236** (23%)

Interleukin-1 (10 ng) was injected on three alternate days. 5 days later, patellae ($n=5$) were dissected and labelled with 40 μCi ^{35}S-sulfate for 4 h.

[a] After thorough rinsing, patellae were either counted directly or cultured for another 48 h in RPMI supplemented with 0.5 μg/ml hr IGF-1.

Loss of incorporated radiolabel in the 48 h time interval is indicated in parentheses. In a second experiment, ^{35}S-PG loss was 46% for contralateral joints, and 25% for the IL-1-treated joints.

p-values compared with its proper control (0 h chase) (Mann–Whitney): * $p<0.001$, * $p<0.01$.

Table 6. Reduced breakdown of proteoglycans synthesized in the recovery phase *in vivo*.

Days after ^{35}S[a]	Days after IL-1[b]	^{35}S-sulfate content	
		Contralateral (CPM ± SD)	IL-1 injected (CPM ± SD)
1	4	929 ± 253	1713 ± 255
3	6	573 ± 119* (38%)[c]	1424 ± 417 (17%)
7	10	437 ± 106** (53%)	1153 ± 241* (33%)

[a] ^{35}S-sulfate (150 μCi) was injected intraperitoneally one day 3 after the IL-1 treatment, $n=6$ per group.

[b] IL-1α was injected on three alternate days. This caused a 41% loss of ^{35}S-prelabelled proteoglycans ($p=0.125$), and a significant inhibition of PG synthesis of 50% on day 1 after the last injection.

[c] Percentage in parentheses represents the cumulative loss of ^{35}S-labelled proteoglycans as calculated from the ^{35}S-sulfate content on day 4 after IL-1 injection. In a second experiment, ^{35}S-PG loss was 42% for the contralateral joints and 26% for the IL-1-treated joints between day 4 and 10.

p-values compared with the contralateral joints or the IL-1-injected joint on day 4 (Mann–Whitney): * $p<0.05$; ** $p<0.01$.

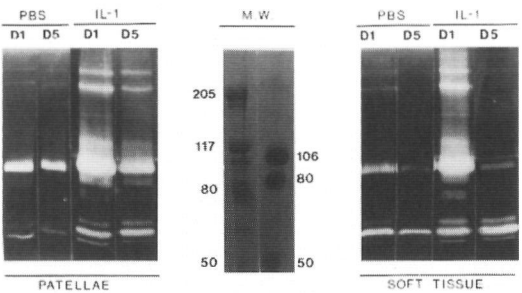

Fig. 4. Zymography of cacodylate extracts of patellae and joint capsules (soft tissue). Interleukin-1α (10 ng) or phosphate-buffered saline (PBS) was injected intra-articularly on three alternate days. Proteoglycan synthesis, 1 day (D 1) and 5 days (D 5) after the last IL-1 injection were 0.53 times and 1.89 times the saline-injected group, respectively. Extracts were normalized based on the dry weights of the tissues. Clear bands on the dark gel indicated gelatinolytic activities. Two middle lanes: prestained molecular weight markers.

Discussion

The objective of this study was to analyze the reparative responses of articular cartilage after an arthritic insult as

induced by intra articular injections of IL-1 into murine knee joints IL-1 was used for a number of reasons first, we recently demonstrated that IL-1 plays a key role in cartilage destruction in several experimental arthritis models in mice [6, 7], second, IL-1 was rapidly cleared from the joint, third, IL-1 caused only a mild and transient joint inflammation [4] Detailed analysis of initial cartilage proteoglycan depletion was performed to provide a basis to study the reparative response

A single IL-1 injection increased the degradation of ^{35}S-PG loss by 1 55-fold within the first 24 h Three repeated IL-1 injections did not further increase the rate of loss, but rather extended the time period during which the cartilage was in a catabolic state We measured an additional loss of 36% over normal Although both newly synthesized and the more processed proteoglycans were as sensitive for IL-1-induced degradation, the measured loss of unlabelled glycosaminoglycans was only 20% This underestimates cartilage depletion since only the superficial layer, physically unseparable from the calcified cartilage layer, became depleted after IL-1 exposure [4, 5] IL-1 induced suppression of chondrocyte proteoglycan synthesis also contributes to the cartilage proteoglycan depletion With the use of radiolabels and electrophoretic separation, we demonstrated that the synthesis of chondroitin sulfate was suppressed, whereas the synthesis of hyaluronic acid chains, aggrecan backbone, was not affected This further indicates that polysaccharide synthesis in general was not affected Benton and Tyler [20], using explants of pig articular cartilage, and Morales and Hascall [21], using bovine articular cartilage, showed that IL 1 down-regulated proteoglycan synthesis at the level of core-protein This suggests that the synthesis of core-protein and chondroitine sulfate chains were linked Total chondrocyte protein synthesis was not affected by IL-1 and we found no dramatic changes which could be used as markers for IL-1 signalling of chondrocytes Analysis of the first phase, the catabolic phase set the parameters to be studied in the recovery phase proteoglycan synthesis and degradation

Articular cartilage fully recovers from the IL-1-induced arthritic insult within 7 days after the last IL-1 injection Recovery was accelerated by an overshoot to enhanced chondrocyte proteoglycan synthesis for a prolonged period of time In rabbits it was also demonstrated that part of the driving force to recuperate from an IL-1 insult was an overshoot to enhanced PG synthesis [22] Proteoglycan recovery after an IL-1 insult occurred earlier in mice than in rabbits This is consistent with the high rate of cartilage proteoglycan turnover (ca 10 15% per day) in young mice, and the high chondrocyte content per wet weight of cartilage ($1\,75 \pm 0\,76 \times 10^5$ cells per 70 μg wet-weight per patella) compared with other species [23] Recovery of the cartilage of rats after an IL-1 insult was not accompanied by supranormal PG synthesis Moreover, the ability to recover was impaired after a second IL-1 injection [24] This was probably due to the much higher IL-1 dose used in rats (150 μg) as compared to our 10 ng injection in mice We found that the recovery was not impaired It even reached significantly higher synthesis at an earlier time point after the last of the three IL-1 injections as compared to a single injection

In vivo replenishment of PGs after papain-induced depletion was preceded by an increased activity of chondrocyte oxidative metabolism (2-fold) and an increased activity of uridine diphosphoglucose dehydrogenase (UDPGD) (1 5-fold), an enzyme directly linked to PG synthesis [25] This may also provide part of the driving force for the supernormal PG synthesis in the reparative phase of IL-1-affected cartilage

In addition, we found evidence for a second, highly relevant recovery mechanism, which may substantially contribute to the replenishment of articular matrices after an IL-1 insult Degradation of proteoglycans was significantly decreased during the recovery phase and was reduced to rates of about 50% of those found in normal cartilage To our knowledge, this novel mechanism has not been described before In normal murine costal cartilage, two populations of ^{35}S-labelled, newly synthesized proteoglycan monomers are found, a major one (85%) of large hydrodynamic size and a minor one (15%) of small hydrodynamic size with half lives of 180 and 247 h, respectively [17] It was interesting to find that the calculated half-lives of ^{35}S-labelled proteoglycans in normal patellae and from patellae in the IL 1 recovery phase were 144 and 216 h, respectively We found no evidence that the impaired PG breakdown was related to a shift towards predominant synthesis of small hydrodynamic size PG monomers The newly synthesized PGs (95%) in the recovery phase have a hydrodynamic volume identical to that of proteoglycans either newly synthesized or preexisting in normal, untreated cartilage A 48 h chase period did not shift the PG monomers synthesized in the recovery phase from large to small hydrodynamic size (processing)

Decreased degradation of proteoglycans could be a result of reduced enzymatic activity The preferable cleavage site of many enzymes, including stromelysin, collagenases and the gelatinases (72 and 95 kDa) is between the G1 and G2 domain of the core-protein of proteoglycans [26, 27] It remains to be defined which enzyme is responsible for the cleavage of proteoglycans Based on sequence data, the IL-1-induced cleavage of core-protein appears not to be at one of the above-mentioned or other known enzymes [28] For this, we examined extracts from tissues of the joint capsule, patellae and articular cartilage by zymography Using copolymerized gelatin as a substrate allowed us to detect a broad range of enzymes, e g, gelatinases, collagenases, cathepsins, plasmin, and stromelysin On day 1 after the third IL-1 injection, all articular tissues expressed enhanced gelatinolytic activities and the 97 kDa protein became the dominant gelatinase Lefebre et al [29] reported that IL-1 enhanced expression of proMMP9 (91 kDa type IV collagenase) and 55 kDa, either proMMP3 and/or proMMP1 (57 kDa interstitial type 1 collagenase) in rabbit articular chondrocytes In the recovery phase, gelatinolytic levels subsided but were still above baseline This did not correlate with the markedly impaired degradation of the newly synthesized proteoglycans Two possibilities exist first, the gelatinases were not responsible for the PG loss, although capable of doing so, second, gelatinase activity was antagonized by inhibitors, e g, tissue inhibitor of metalloproteinases (TIMP) [30]

In this study, changes in proteoglycan synthesis and degradation coincided in both phases of the IL-1-induced insult This does not necessarily have to mean that both processes were linked Arner and Pratta [31] found in IL-1-challenged bovine nasal cartilage, that both processes were mediated by independent post-receptor mechanisms We demonstrated that the recovery of IL-1-induced arthritic insult in mice was facilitated by enhanced proteoglycan synthesis and impaired degradation of proteoglycans Studies are in progress to understand the mechanism of decreased proteoglycan degradation

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CHAPTER 5

PROTECTION AGAINST CARTILAGE PROTEOGLYCAN SYNTHESIS INHIBITION BY ANTI-INTERLEUKIN-1 ANTIBODIES IN EXPERIMENTAL ARTHRITIS

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Protection Against Cartilage Proteoglycan Synthesis Inhibition by Antiinterleukin 1 Antibodies in Experimental Arthritis

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Abstract. **We have used neutralizing antibodies raised against murine recombinant interleukin 1 (IL-1) to demonstrate a role for IL-1 in the cartilage destruction and inflammation of antigen induced arthritis. *Ex vivo* production of IL-1 was demonstrated in tissue cultures of joint cross sections shortly after arthritis induction. Neutralizing antimurine IL-1 antibodies identified the activity to be about 80% IL-1α 24 h after onset of arthritis. In animals receiving a single injection of anti-IL-1 antisera at Day −3, cartilage proteoglycan synthesis suppression during the first 2 days of arthritis was prevented. Normal proteoglycan synthesis was maintained until Day 4 when anti-IL-1 antisera was given at Days −2, 0, and 2 of arthritis. Dose response experiments showed that the reduction in inflammation was insufficient to account for the clearcut reduction in cartilage proteoglycan synthesis inhibition. Our results demonstrate that IL-1 plays a role in cartilage pathology in murine antigen induced arthritis. (*J Rheumatol 1992,19:348-56*)**

Key Indexing Terms.
INTERLEUKIN 1 EXPERIMENTAL ARTHRITIS CARTILAGE
INFLAMMATION BLOCKING STUDIES MICE

A central role of interleukin 1 (IL-1) in the pathology of rheumatoid arthritis (RA) has been proposed, because it can be produced by and has effects on numerous cell types present in the arthritic joint[1,2].

It is known that IL-1 has profound catabolic effects on articular cartilage. This has been demonstrated *in vitro* on living cartilage explants derived from different species, including murine patellar cartilage[3-5]. Moreover, intraarticular injections of IL-1 in rabbits[6-8], rats[9], and mice[10] resulted in marked depletion of cartilage matrix, a predominant influx of neutrophils into the joint, and synovitis similar to that seen in experimental arthritis. It has also been reported that IL-1 could modulate immune responses associated with the arthritic process. Systemic IL-1 can accelerate onset and increase incidence of collagen type II arthritis in DBA mice[11,12], and may potentiate spontaneous arthritis in MRL mice[13]. Intraarticular IL-1 injections reduced antigen methylated bovine serum albumin (mBSA) induced swelling and histopathology of joints of immunized rats[14], but potentiated the effects of antigen induced inflammation in nonsensitized mice[15]. In the chronic phase of both antigen induced arthritis (AIA) and peptidoglycanpolysaccharide induced arthritis, intraarticular IL-1 injection induced exacerbation of the smouldering inflammation[16,17].

Finally, IL-1 has been reported to be present in synovial fluids of patients with RA[18].

These observations fulfill criteria for IL-1 to be a potential mediator in arthritis, and suggest it may have a role in the disease process. These studies do not, however, provide direct evidence for IL-1 involvement as a mediator of arthritis. Moreover, other cytokines, such as TNF and IL-6, also fulfill these criteria to some extent[19-22]. It is therefore imperative that direct methods are used to determine if IL-1 has a role in arthritis.

Using antibodies directed against murine recombinant IL-1, we have demonstrated that in murine antigen induced arthritis, synovial tissue releases IL-1 and that the neutralizing antibodies block the cartilage changes and partially inhibit the acute inflammation of antigen induced arthritis (AIA). These data provide the first direct evidence that IL-1 plays a role in cartilage pathology of AIA.

MATERIALS AND METHODS

Induction and treatment of experimental arthritis C57b1/6 mice, aged 6-8 weeks at the start of the experiments, were immunized with mBSA (Sigma Chemical Company, St Louis, MO, USA) as described[23]. Arthritis was induced 21 days after the start of the immunization by injecting 60 μg mBSA in phosphate buffered saline (PBS) into the left knee joint.

Assessment of joint inflammation Joint inflammation was measured by ^{99m}Technetium (^{99m}Tc) pertechnetate uptake in the knee joints[24]. Briefly, animals were sedated by intraperitoneal administration of 4.5% chloral hydrate, 0.1 ml/10 mg of body weight. About 10 μCi ^{99m}Tc in 0.2 ml saline was injected subcutaneously in the neck region. After 15 min the accumulation of the isotope in the knee due to increased blood flow and tissue swell-

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ing was determined by external gamma counting. The severity of inflammation was expressed as the ratio of the ^{99m}Tc uptake in inflamed over contralateral knee joint.

Assessment of proteoglycan synthesis. Patellae (5 or 6 pieces), in a minimal amount of adjoining soft tissue, were placed in a 2 ml RPMI-HEPES medium (Flow Laboratories, Irvine, Scotland) with gentamicin (50 μg/ml), and L-glutamine (2 mM) and 40 μCi ^{35}S-sulfate. At the end of the 3 h incubation period, patellae were fixed in 10% formalin and subsequently decalcified in formic acid (5%) for 4 h. Patellae were punched out of adjacent tissue, and dissolved in 0.5 ml Luma solve (Hicol, Oud-Beijerland, The Netherlands). The ^{35}S content of each patella was measured by liquid scintillation counting.

Histology of knee joints. The left and right joints were dissected and processed for histology as described[23]. Standard frontal sections (7 μm) were prepared and stained with safranin-O, and depletion was visualized by diminished staining of the cartilage matrix.

IL-1 production by synovial tissue. A cross section of synovial tissue was taken ventrally at the knee joint. It contains, in addition to the synovia, the patella and adjoining tendon. Synovial tissue comprised of specimen from 6 knees to exclude variation between specimens was incubated in 2 ml RPMI medium at 37°C in a CO_2 incubator for 1, 6, and 24 h. Dilutions of these culture media were tested for IL-1 activity by a sensitive bioassay based on the IL-1 specific EL-4 NOB-1 line in conjunction with the IL-2 responder CTLL line. More IL-1 bioactivity was detected in 1 h than in prolonged tissue incubations. Recovery of exogenously added IL-1 to the supernatant was 85% and 60% in the 1 h and 24 h tissue incubations, respectively. This was not due to cytotoxicity of the culture media, because viability and cell numbers in the bioassay were unchanged.

Assessment of protein levels. Protein-levels of synovial tissue conditioned media were measured by a colorimetric method based on the coomassie brilliant blue G250 color shift according to the manufacturer's procedure (Bio-Rad Protein-assay kit I, Bio-Rad Laboratories).

Bioassay for IL-1. IL-1 activity was measured in the one-stage bioassay for IL-1 as described by Gearing, *et al*[25]. In short, the assay was performed as a coculture of the IL-1 specific subclone of the murine thymoma cell line EL-4, designated NOB-1 producing IL-2 and IL-4 with the lymphokine responder CTLL line. The cells were plated out in concentrations of 1×10^5 well NOB-1 cells and 4×10^3 well CTLL cells in RMPI supplemented with 10% fetal calf serum; after 18 h 0.5 μCi/well of ^3H-TdR was added and incorporation was measured after 3 h. Samples were tested in triplicate, and in a monoculture of CTLL line to exclude possible IL-2 impurities of the samples. The sensitivity limit of the NOB-1 assay was less than 0.1 pg/ml, 200 × more sensitive than the original leukocyte associated function assay. Titration of IL-1 showed that the NOB-1 line was almost as sensitive to mrIL-1α as to mrIL-1ß (Figure 1A), with half-maximal proliferation at about 1 pg/ml.

Antiserum treatment of mice. Polyclonal rabbit antimurine IL-1ß antiserum and polyclonal goat antimurine recombinant IL-1α antiserum were prepared by immunization with complete Freund's adjuvant against cloned, purified biologically active mature IL-1α[26] and IL-1ß[27]. Anti-IL-1α antiserum was 8 × more potent in neutralizing IL-1 in the NOB-1 assay than anti-IL-1ß antiserum (Figure 1B), and showed no cross reactivity (not shown). Each of the preparations of antibodies (anti-IL-1α or anti-IL-1ß) was tested *in vitro* and showed no neutralizing crossreactivity against IL-6, tumor necrosis factor (TNF), and granulocyte macrophage colony stimulating factor (unpublished). Western analysis and intracellular labelling of macrophages further subscribed the specificity of anti-IL-1 antibodies[28,29]. For *in vivo* treatment, mice were injected sc with 200 μl of a 1:1 mixture of both antisera, and administered at various times relative to the induction of arthritis as described in Results. In all experiments, 200 μl of a 1:1 of goat and rabbit normal sera was administered to groups of mice to control for nonspecific effects of sera.

Purification of anti-IL-1 antiserum by chromatographic separation. The initial step in the IgG purification process of the sera was a limited precipitation of proteins with 45% ammonium sulfate in serum. The precipitate was spun down, and the pellet was redissolved and dialyzed against bidistilled water at 4°C.

Immunoglobulins from the rabbit anti-IL-1 antiserum were purified by affinity chromatographic separation on a protein A sepharose CL4B column. Serum proteins were present in the void volume, and immunoglobulins were separated from the column with the elution buffer, 0.1 M glycine-HCl, pH 2.5. Fractions were dialyzed against bidistilled water and concentrated down to the starting point volume. The protein fractions were added back to the dialyzed ammonium sulfate supernatant.

Immunoglobulins from the goat anti-IL-1 antiserum were purified by ion exchange chromatography on a DEAE-Trisacryl M column. Immunoglobulins were detached from the column by low salt elution, 0.025 M Tris-HCl, 0.035 M NaCl, pH 8.8, and the other serum proteins were isolated from the column by the high salt elution buffer, 0.1 M Tris-HCl, 1 M NaCl, pH 8.8. Column fractions were dialyzed against bidistilled water, concentrated to the original volume. The second fraction was added back to the dialyzed ammonium sulfate supernatant.

All the individual fractions were tested for anti-IL-1 antibody by ELISA, and this demonstrated that the chromatographic separation of anti-IL-1 antibodies was absolute.

RESULTS

IL-1 production by synovial tissue from inflamed joints. Synovial tissues were removed from knee joints of normal and AIA mice and examined for their ability to release IL-1 into the culture supernatant. Preliminary experiments had shown that 1 h incubation was sufficient to obtain a significant release of IL-1 activity from AIA, but not normal tissue. Therefore, all tissue samples were incubated for 1 h in tissue culture medium and the supernatant assayed for IL-1 using the NOB-1 assay.

A distinct IL-1 signal was obtained 3 h after induction of arthritis. Time course studies showed further increases at 6 and 12 h and maximal production at 24 h (Figure 2). IL-1 levels declined thereafter. The IL-1 released from tissues of untreated joints, or inflamed joints on Day 4, were below the detection limit of 0.1 pg/ml. IL-1 production could also be induced by mBSA in nonimmune mice, although to a low extent (Figure 2). In several experiments, the estimated amount of IL-1 released during the 1 h incubation was 10–30 pg/synovia, using tissue from 24 h AIA, whereas only 1–2 pg was released by tissue from mBSA injected, nonimmune mice. Conditioned media of noninflamed tissue contained 0.16 ± 0.07 mg protein ($n = 10$ different conditioned media samples). Protein levels in culture media of inflamed tissues taken from Day 1 of AIA were twice that high. Correction of IL-1 levels for total protein content of conditioned media still resulted in significant IL-1 levels of 5–15 pg.

Blocking by specific antiserum demonstrated that about 80% of the NOB-1 activity released from the tissue at Day 1 of arthritis could be ascribed to IL-1α (Figure 3). IL-1 levels in serum were not significantly elevated at any time point (not shown). Our results demonstrate that local IL-1 production is induced in articular tissue immediately after the onset of arthritis.

In vivo demonstration of the neutralizing capacity of the anti-

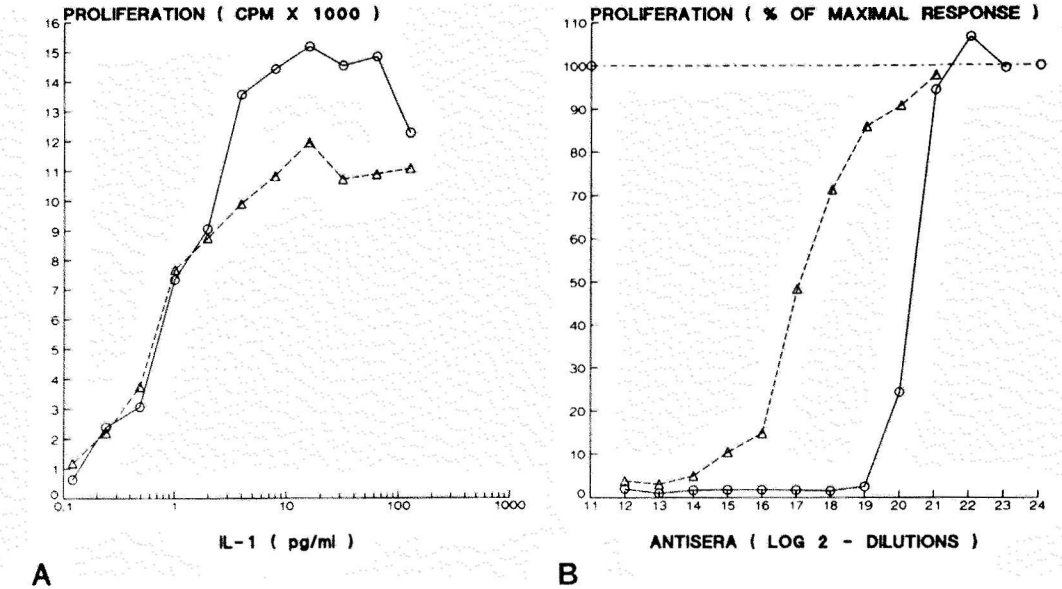

Fig. 1. Titration of IL-1 and anti-IL-1 antiserum in NOB-1 assay. (A) Dose dependent effect of mrIL-1α (o—o) and IL-1ß (Δ -- Δ) on CTLL cells cocultured with EL4-NOB-1 cells for 24 h. Proliferation response was measured by ^{3}H TdR incorporation and expressed as CPM values. (B) IL-1 standards (31 pg/ml) were preincubated for 1 h with different dilutions of anti-IL-1 antiserum. The residual IL-1 activity was measured in the NOB-1 assay and expressed as a percentage of maximal proliferation (IL-1α, 11,270 cpm; IL-1ß, 15,955 cpm).

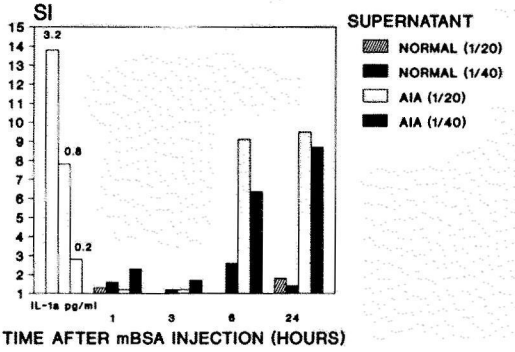

Fig. 2. IL-1 released by synovial tissue *ex vivo*. Patellae with adjoining soft tissue were dissected at different times after intraarticular injection of mBSA (60 μg) in normal or mBSA preimmunized mice. Six specimens were incubated in 2 ml RPMI medium for 1 h and dilutions (1/20 and 1/40) of the media were tested in triplicate for IL-1 activity in the NOB assay. Titration of mrIL-1α served as a control. Proliferation expressed as stimulation index (SI). ^{3}H-TdR incorporation, background 2000 cpm; maximum 27,737 cpm.

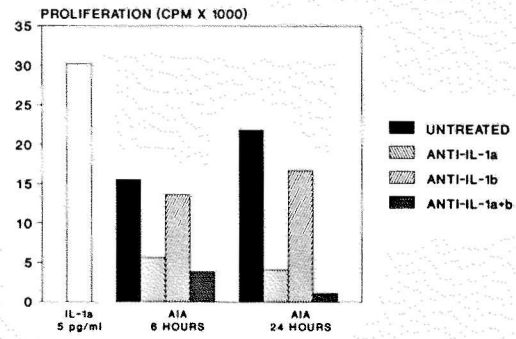

Fig. 3. Identification of IL-1 in the culture supernatants by neutralizing antibodies. Culture supernatant of joint tissues taken 6 and 24 h of AIA was incubated in the presence of (1/8000 diluted) neutralizing antiserum to IL-1α (AIL-1a) and IL-1ß (AIL-1b) or a combination of both (AIL-1a+b). Methods as described in Figure 1.

IL-1 antisera. The *in vivo* effectiveness of the anti-IL-1 antiserum was tested by examining its ability to neutralize intra-articularly injected IL-1. Injections of IL-1 into the joint cavity induced a significant suppression of proteoglycan synthesis by patellar cartilage. Kinetics revealed that anti-IL-1 antisera given shortly before 1 or 10 ng of IL-1 blocked the IL-1 suppression of proteoglycan synthesis significantly (Figure 4). Inhibition of high dose intraarticular IL-1ß and IL-1α (10 ng) was further enhanced by extending anti-IL-1 antisera treatment of mice to Days −2, and −1. This shows

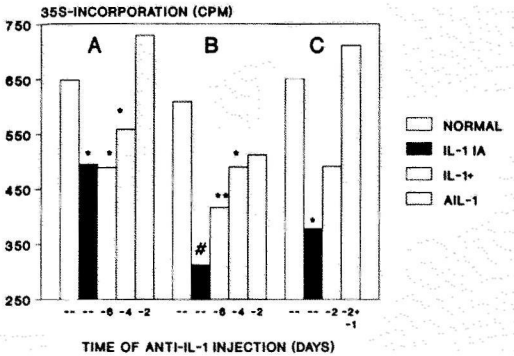

Fig. 4. Prevention of IL-1 induced proteoglycan synthesis suppression by *in vivo* administration of antiserum to mrIL-1. IL-1 was injected intraarticularly; IL-1ß, 1 ng (A); IL-1ß 10 ng, (B); IL-1α 10 ng (C). Proteoglycan synthesis at Day 2 was measured *ex vivo* by ^{35}S-sulfate incorporation as described in Materials and Methods. Mice received 100 µl anti-IL-1 antiserum (AIL-1) sc at Days 6, 4, 2 and/or 1 before IL-1 injection. Statistical significance was calculated with Student's t test; * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$, from proteoglycan synthesis of IL-1 injected compared to healthy knee joints.

that the anti-IL-1 antisera has sufficient activity to neutralize locally synthesized IL-1, and is suitable for exploring the role of IL-1 in AIA.

Effect of anti-IL-1 antisera treatment on arthritis. A subcutaneous injection of 200 µl of anti-IL-1 antisera, or control sera at Day −3, induced a slight reduction in edema as measured by decreased ^{99m}Tc uptake of the inflamed joint at Day 2 (Table 1). The mean reduction in edema by anti-IL-1 antisera treatment from 4 replicate experiments was 12 ± 10% at Day 2. On Day 2 of arthritis, proteoglycan synthesis in patellae was about 55 ± 21% of normal value. Anti-IL-1 antisera treatment significantly maintained the proteoglycan synthesis at 98 ± 23% of the normal synthesis (mean of 4 separate experiments). On Day 4 of arthritis, a significant reduction in edema was measured after anti-IL-1 antisera treatment, but the proteoglycan synthesis was slightly suppressed (Table 1). Histology of the whole arthritic knee joint at Day 4 showed depletion of articular cartilage, and a single injection of anti-IL-1 antisera at Day −3 did not improve the outcome (not shown). Moreover, treatment started 2 days after AIA induction was insufficient to abrogate suppression of chondrocyte proteoglycan synthesis (57%, $p<0.001$) and joint inflammation (1.68 ± 0.41) at Day 4. To determine whether this was due to a depletion of anti-IL-1 antibody or whether IL-1 antisera treatment was no longer important in Day 4 arthritis, the anti-IL-1 antisera treatment was prolonged; animals were injected sc at Days −2, 0, and 2 of arthritis. The anti-IL-1 antisera treatment induced a consistent reduction of edema at Day 2 (22 ± 18%), and Day 4 (29 ± 7%). Now, in addition, there was a significant prevention of the inhibition of ^{35}S-proteoglycan synthesis at Days 2 and 4 of arthritis (Table 2). The mean proteoglycan synthesis from 3 replicate experiments went from 47 ± 9% of the normal value to 98 ± 22% at Day 4 of arthritis. Control serum reduced the inflammation by 8 ± 15% at Day 2 and by 12 ± 18% at Day 4, but this had no beneficial effect on cartilage metabolism.

Effect of purified anti-IL-1 antibodies. To demonstrate that the active principle in the anti-IL-1 antisera could be ascribed to anti-IL-1 antibodies, we purified the immunoglobulins and performed a comparative study with IgG depleted serum and irrelevant antibodies. Treatment was started 24 h prior to induction of antigen-induced arthritis. Normal serum, purified antibodies against the irrelevant antigen lysozyme, and serum devoid of immunoglobulins had no effect on the proteoglycan synthesis suppression at Day 2 of arthritis. Complete anti-IL-1 antiserum and the purified anti-IL-1 antibodies prevented the proteoglycan synthesis suppression to full extent (Figure 5). This result demonstrated that anti-IL-1

Table 1. *Effect of a single injection of anti-IL-1 antibodies on AIA*

Treatment	Time	Tc Uptake (L/R)	Proteoglycan synthesis‡ Control	Arthritic	
—	Day 2	1.95±0.29	932±218	632*±152	68%
Normal sera	Day 2	1.94±0.18	842±102	622**±90	74%
Anti-IL-1	Day 2	1.68±0.30	900±104	1078±191	120%
—	Day 4	1.65±0.53	992±112	631*±173	64%
Normal sera	Day 4	1.51±0.31	1001±81	477†±83	46%
Anti-IL-1	Day 4	1.21*±0.07	1085±340	770±273	71%

Representative of 4 experiments. Each value represents the mean ± SD calculated from groups of 5 or 6 mice. Mice were injected sc 3 days before induction of arthritis with 200 µl of normal or anti-IL-1 antiserum.

Edema was expressed as ratio of ^{99m}Tc uptake by inflamed joint (L) over the contralateral joint (R). A value near 1.0 is typical for untreated mice.

‡ Proteoglycan synthesis was measured *ex vivo* by $^{35}SO_4$ incorporation (CPM), and expressed as a percentage of contralateral patellae. The statistical significance of differences from control ^{35}S incorporation was calculated with Student's t test, and the statistical significance of the ^{99m}Tc ratios with the Wilcoxon rank sum test; * $p < 0.05$; ** $p < 0.01$; † $p < 0.001$.

Table 2. *Prolonged treatment of arthritic mice with antibodies directed against IL-1*

Treatment	Time	Tc Uptake (L/R)	Proteoglycan Synthesis Control	Arthritic	
—	Day 2	2.27±0.50	876±225	486*±101	55%
Normal sera	Day 2	2.42±0.32	852±108	480†± 69	56%
Anti-IL-1	Day 2	2.32±0.46	861±152	817 ±153	95%
—	Day 4	1.61±0.15	856± 92	395†± 85	46%
Normal sera	Day 4	1.32†±0.13	722± 61	468†± 29	65%
Anti-IL-1	Day 4	1.20†±0.17	680± 72	833*±133	123%

Treatment consisted of sc injections at Days −2, 0, and 2 of arthritis. Arthritis was induced with 60 μg mBSA, Day 2 and Day 4 were examined. Methods and statistics as described in legend of Table 1.

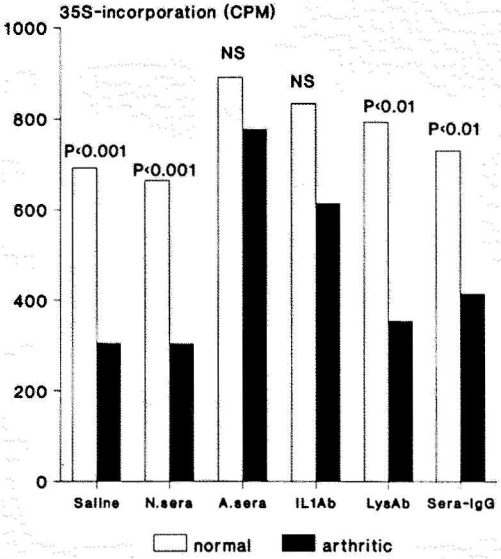

Fig. 5. Prevention of proteoglycan synthesis suppression in arthritis by purified anti-IL-1 antibodies. Mice were injected intraperitoneally with 200 μl anti-IL-1α+ß antisera (A.sera) or an equivalent of anti-IL-1α+ß antibodies (IL1Ab) at 24 h before induction of AIA. As a control, mice were treated with saline, antilysozyme antibodies (LysAb), or normal complete sera (N.sera). Patellar proteoglycan synthesis was measured at Day 2 of arthritis. Methods and statistics as described in legend of Table 1.

antibodies in the antiserum were responsible for the observed effects on cartilage.

Direct interference with IL-1 action on cartilage. Dose response experiments showed that the reduction in inflammation exerted by the anti-IL-1 antibodies could not account for the complete prevention of articular proteoglycan synthesis suppression (Table 3). The lower antigen dose elicited significantly less joint inflammation, as reflected by Tc uptake, but nevertheless the suppression of proteoglycan synthesis inhibition in the articular cartilage was in the same order of magnitude as that obtained with the high antigen dose. Again, anti-IL-1 antibody treatment reduced both the inflammation and proteoglycan synthesis suppression.

Histology further confirmed the protective effect of anti-IL-1 antibodies on cartilage pathology. Whole knee joint sections revealed marked proteoglycan depletion in the arthritic cartilage of the control group and clear cut reduction of this depletion in the anti-IL-1 group. Moreover, less chondrocyte death occurred in the latter group (Figure 6).

Taken together, our results show that IL-1 is a key mediator in cartilage destruction in the early stage of AIA in mice.

DISCUSSION

We set out to obtain direct evidence for the involvement of IL-1 in AIA in mice. As many studies have demonstrated that IL-1 has effects *in vitro* and *in vivo* consistent with a role in arthritis, we felt that 2 additional criteria needed to be fulfilled before we could declare that IL-1 has a significant pathogenic role: first, that IL-1 is present at the disease

Table 3. *Prevention of proteoglycan synthesis suppression is independent of decreased joint inflammation*

Treatment	Dose	Tc Uptake (L/R)	Proteoglycan Synthesis Control	Arthritic	
—	20 μg	1.28±0.20	690± 76	377†± 72	55%
—	60 μg	1.45±0.27	712±127	457†± 76	64%
Anti-IL-1	60 μg	1.27±0.24	824±128	675 ±167	82%

Treatment consisted of sc injections at Days −2, 0, and 2 of arthritis. Arthritis was induced with 20 or 60 μg mBSA and was examined on Day 4. Methods and statistics as described in legend of Table 1.

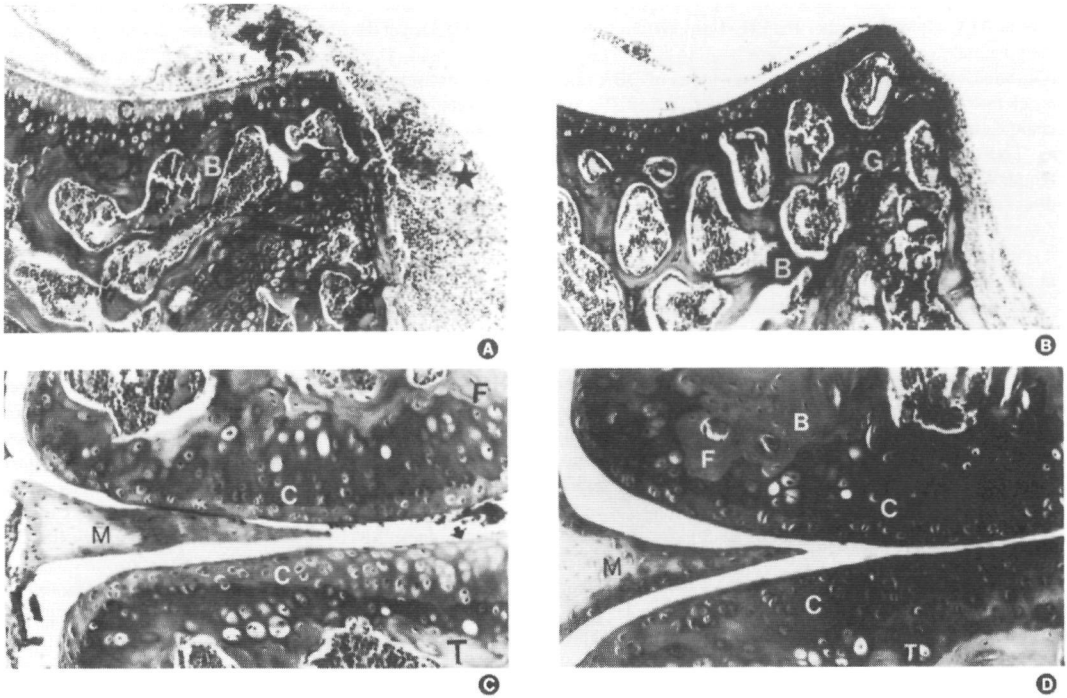

Fig. 6. Frontal sections of knees from Day 4 of arthritis stained with safranin-O. Cartilage proteoglycan depletion in the femoropatellar joint (patella is removed) in the arthritic mice (A). Cells in infiltrate and exudate are predominantly PMN (*). Prolonged treatment with anti-IL-1 antisera, 3 sc injections at Days −2, 0, and 2 of arthritis, resulted in reduced cartilage depletion (B). Higher magnification of the femorotibial joint showed proteoglycan loss and the presence of pycnotic chondrocytes (C, arrow). Note the absence of these characteristics of arthritis after prolonged treatment with anti-IL-1 antisera (D). Abbreviations: B, bone; C, cartilage; F, femur; G, growth plate; M, meniscus; T, tibia.

locus, and second, that inhibition of IL-1's effects would lead to an amelioration of the disease.

Because of the impracticability of obtaining synovial fluid (SF) from a mouse, we elected to determine if IL-1 was released from joint tissue. We used a short 1 h incubation time so that measurements would be made of ongoing synthesis rather than synthesis induced by tissue treatment. Thus, within 1 h of LPS stimulation, murine macrophages did not produce significant amounts of IL-1 by Western analysis. Culture for 1 h of joint cross sections dissected from arthritic joints showed substantial release of IL-1 during the first 2 days of AIA (Figure 2). Tissue taken at later time points or from normal joints failed to release detectable IL-1. IL-1 and protein levels were not related because total protein levels in the culture media of inflamed tissues were doubled compared to noninflamed synovial tissues from Day 1 to Day 7 of AIA. Thus IL-1 production coincides with the acute onset of inflammation and precedes the depletion of articular cartilage.

We had found that intraarticularly injected IL-1 causes a marked suppression of proteoglycan synthesis in mice within 24 h[10,30]. This could account for the observed inhibition of proteoglycan synthesis at Day 2 of arthritis. This implies a link between IL-1 synthesis and the pathology of articular cartilage.

We identified the synovial tissue released IL-1 activity to be primarily IL-1α by specific antibody blocking (Figure 3). It has been reported that both IL-1α and IL-1ß are present in the SF of patients with arthritis[18]. IL-1ß is in part released in an inactive precursor form[28,31], and may not be detected in the NOB-1 bioassay. In tissue culture of murine macrophages, we find that IL-1ß is secreted rapidly and completely within 24 h of stimulation by LPS, whereas IL-1α continues to be released for at least 96 h after stimulation (unpublished). Our results should therefore be taken as definitive evidence that IL-1 is present. However, it does not indicate either absolute or relative amounts of IL-1α or IL-1ß in the arthritic joint. Further studies on this subject are ongoing.

Having demonstrated that IL-1 is present in the arthritic

tissue, the next question was whether it plays a pathological role in AIA Cytokines other than IL-1 are known to have the potential to cause arthritis Transforming growth factor-ß injected intraarticularly into the knee joints of DBA mice accelerated the onset of type II collagen arthritis[32], and could cause arthritis directly in rats[33] Other mediators such as TNFα[19], and some as yet uncharacterized T cell lymphokines[34] have all been shown to cause arthritis upon direct intraarticular injection Moreover, evidence has been presented that most of these cytokines are present in SF Thus it is important to prove whether there is a direct role for IL-1 in the pathogenesis of AIA

Several methods are available to directly block the activity of IL-1 with sufficient specificity to determine whether IL-1 plays a pathologic role in cartilage destruction A monoclonal antibody directed against the IL-1 receptor, which blocks the action of IL-1, although effective in other diseases[35], may not be useful here because immunoglobulins do not easily penetrate articular cartilage[36] An IL-1 receptor antagonist (IL 1ra) has been isolated, cloned and sequenced, which antagonizes IL-1 action *in vitro* and *in vivo*[37] Cartilage is accessible for IL-1ra because of its size of 23–26 kDa Nevertheless, it is difficult to obtain good inhibitor levels in articular cartilage at the chondrocyte receptor because it has a short half-life ($t_{1/2}$) and inability to accumulate in cartilage

In addition, there is a soluble form of the IL-1 receptor that binds to IL-1 and thereby may prevent its binding to cell bound receptors[37] A short $t_{1/2}$ (2 h) may limit its use in chronic studies[38] We chose to use polyclonal neutralizing antibody for our studies in chronic AIA because they have an *in vivo* $t_{1/2}$ of 7 days and because we had been able to document complete blockage of *in vivo* IL-1 effects on cartilage (Figure 4) Unlike antireceptor antibody, it does not need to penetrate the cartilage to be effective

We found in AIA that specific elimination of IL-1 by polyclonal anti-IL-1 antibodies resulted in significant suppression of edema, diminished influx of inflammatory cells and maintenance of chondrocyte proteoglycan synthesis Histologic sections demonstrated that treatment of mice with anti-IL-1 antiserum prevented cartilage loss and chondrocyte death, both typical pathological sequelae of antigen induced arthritis

The effects of the anti IL-1 antisera on inflammation and on cartilage metabolism must be regarded as independent phenomena Multiple doses of normal sera could suppress the inflammation, but could not prevent the suppression of chondrocyte proteoglycan synthesis When anti-IL-1 antisera was given as a single dose 3 days before disease induction, the nonspecific effect of serum injection was absent, yet a significant inhibition of inflammation by anti-IL-1 was still observed (Table 1) This indicates IL-1 involvement in the inflammatory process However, when a low dose of antigen (20 μg mBSA) was given, it induced a mild level of joint inflammation (1 28 ± 0 20) at Day 4 of AIA, comparable to that found in the antisera or normal sera treated mice (Table 1), yet the proteoglycan synthesis was still markedly suppressed Therefore, the reduction of inflammation by anti-IL-1 antisera was too small to account for the abrogation of proteoglycan synthesis inhibition This indicates that the antisera directly interferes with IL-1 action on cartilage metabolism We have shown that both *in vitro* IL-1[7] and intraarticular IL-1[10 30] can directly inhibit cartilage proteoglycan synthesis in the mouse We believe this is the first demonstration that during an arthritic process, IL-1 directly asserts this effect on the cartilage

Patellar cartilage breakdown as measured by loss of ^{35}S-sulfate prelabelled proteoglycans is currently under investigation Preliminary results demonstrated that the ≈45% breakdown of proteoglycans from patellar cartilage at Day 2 of arthritis remained unchanged regardless of anti-IL 1 antiserum treatment This implies that the cartilage proteoglycan breakdown is not an IL-1 driven process in both the nonimmunologically mediated zymosan induced arthritis and antigen driven arthritis, AIA The beneficial effects of anti-IL-1 antibody treatment on the diminished cartilage proteoglycan loss as observed with histology indicates that chondrocyte proteoglycan synthesis inhibition contributes to the total proteoglycan loss by insufficient resynthesis

In a previous study, we demonstrated that the normal daily loss of 12% was redoubled once after a single intraarticular injection of 10 ng IL-1α[10] Depletion of the articular cartilage was histologically observed only after repeated IL-1 injections, and the longterm proteoglycan synthesis suppression by IL 1 accounted for at least half the total proteoglycan loss

Therefore, continuous suppression of chondrocyte proteoglycan synthesis by *de novo* synthesized IL-1 during the early phase of experimental arthritis contributes to the overall depletion of cartilage matrix In humans, the proteoglycan turnover rate is 2–5 times slower than in murine articular cartilage, and proteoglycan synthesis inhibition by endogenous IL-1 will only affect the cartilage matrix in the long run

Chondrocyte death is found to be a common accompaniment of experimental arthritis and inflammation The protective effect of anti-IL-1 antisera on this phenomenon was unexpected Decreased inflammation *per se* cannot account for the decreased chondrocyte death as it occurs in the milder 20 μg mBSA arthritis, and in the arthritis where inflammation was suppressed by the administration of normal sera Although cellular cytotoxicity induced by IL-1 has been reported for some cell types[39 40], it has not been reported for chondrocytes It is more likely that IL-1 participates indirectly in eliciting chondrocyte death Mechanisms such as synergy with other cytokines as TNF, potentiation of oxygen radical formation, enhancing cell infiltration or activating inflammatory cells via IL-8 induction, or facilitating release of other toxic mediators may provide a more likely explanation

We have shown that in the acute induction phase of AIA IL 1 plays a major role in proteoglycan depletion by inhibition of proteoglycan synthesis and chondrocyte death These results show that interference with IL 1 can lead to a favorable therapeutic effect on a form of experimental arthritis Because of the many similarities of AIA with human RA, it suggests that IL 1 could also play a major role in the human disease

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CHAPTER 6

MODULATION OF CARTILAGE DESTRUCTION IN MURINE ARTHRITIS WITH ANTI-IL-1 ANTIBODIES

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Modulation of cartilage destruction in murine arthritis with anti-IL-1 antibodies

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Abstract

One of the early events in murine antigen-induced arthritis is the generation of IL-1 in the inflamed joint We investigated the role of IL-1 in the acute phase of the arthritic process by selective blockage of IL-1 bioactivity by treatment with neutralizing antibodies Pretreatment with anti-IL-1 antibodies moderately suppressed joint swelling The decrease in chondrocyte proteoglycan synthesis seen in the acute phase of arthritis was prevented by treatment with anti-IL-1 antibodies IL-1 does not appear to be a major contributor to the accelerated breakdown of articular cartilage in this model The major impact of anti-IL-1 antibodies was the prevention of proteoglycan synthesis inhibition which clearly reduced articular cartilage depletion by maintaining normal proteoglycan synthesis

Introduction

The importance of IL-1 in the arthritic process is based on IL-1's impact on connective tissue integrity, and its pivotal function in humoral and cellular inflammatory processes Furthermore, IL-1 mRNA was found to be present in inflamed synovial tissue [1], and IL-1 levels in sera correlates well with disease activity [2]

Recent attention is focused on the use of cloned, naturally occurring IL-1 inhibitor, IL-1 receptor antagonist (IL-1ra) Although effective in many acute systemic diseases [3], its use in arthritis is hampered for several reasons First, IL-1ra's half-life *in vivo* was only about 30 min, secondly a 1000-fold excess of this inhibitor is commonly required to suppress effectively IL-1's acitivity In order to elucidate the role of IL-1 in antigen-induced arthritis model (AIA), we treated mice with neutralizing antibodies directed against murine recombinant IL-1 The treatment started prior to induction of arthritis, but after a fully developed immune response to the antigen We measured joint swelling, chondrocyte proteoglycan (PG) synthesis and breakdown in the arthritic knee joint

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Materials and methods

Assessment of experimental arthritis

Arthritis was evoked with 60 μg mBSA injected into the right knee joint cavity in sensitized male C57Bl/6 mice Joint swelling was measured externally by enhanced uptake of ^{99m}Technetium pertechnetate of the inflamed knee joint from the circulation, compared with the normal contralateral joint [5] Chondrocyte proteoglycan synthesis

in patellar cartilage was measured *ex vivo* by a 3 h incubation with ^{35}S sulfate enriched RPMI [6] Proteoglycan breakdown was measured as loss of *in vivo* ^{35}S-prelabeled cartilage Cartilage depletion was either quantitated by measuring glycosaminoglycan content of papain-digested patellar cartilage with the Farndale assay [4] or by histological scoring of knee joint sections stained with safranine O

Measurement of cytokine release with bioassays

Patella with adjacent synovial tissue was taken and six specimen were incubated in 2 ml RPMI-medium at 37 C in a CO_2-incubator for 1 h Dilutions of these culture media were tested for IL-1 and IL-6 bioactivity IL-1 activity was measured in the one stage bioassay for IL-1, a coculture of the IL-1-specfic subclone of the murine thymoma EL-4 NOB-1 cell, producing IL-2 with the IL-2 responder CTLL cell IL-6 activity was measured as a proliferative response of B9-cells IL-1 and IL-6 activities were verified by neutralizing antibodies

Antiserum treatment of mice

Rabbit anti-murine IL-1β antiserum, and rabbit anti-murine recombinant IL-1α antiserum were prepared by immunization against cloned, purified biologically active mature IL-1α, and IL-1β emulsified in aluminium hydroxide (Imjectth Alum, Pierce, Rochford, Illinios, USA) Both antisera showed no cross reactivity or neutralizing reactivity against IL-6, IL-4, and IL-2 Antibodies were purified by affinity chromatographic separation on protein A sepharose CL4B column Mice were given 1 mg of purified antibodies or 100 µl of each antiserum intravenously 18 h before induction of arthritis or thereafter

Results and discussion

Cytokine profiles of IL-1 and IL-6 were recorded in wash-outs of joint tissues taken from mice with AIA High levels of IL-1 and IL-6 were released by inflamed tissue taken at day 1 of arthritis (Table 1) By use of neutralizing antibodies we identified IL-1α to account for 80% of the IL-1 bioactivity At day 4, no IL-1 could be detected but IL-6 was still elevated Pretreatment of mice with anti-IL-1 antisera (against IL-1α and IL-1β) completely eliminate IL-1 bioactivity in the joint at day 1 indicating that

Table 1
Effect of anti IL 1 antibody treatment on cytokine levels in wash outs joint swelling and proteoglycan synthesis of patellae

Treatment			IL 1	IL 6	Edema	PG synthesis (CPM ± SD)		*t* test
Source		Start[1]	(pg/ml)	(ng/ml)	Tc ratio	Left	Right	*p*-value
Day 1 AIA								
N Rabbit	Serum	−18 h	156	25 6	2 37 ± 0 20	1372 ± 419	627 ± 282	0 005
AIL 1α + β	Serum	−18 h	< 0 5	25 2	2 55 ± 0 20	1331 ± 350	1070 ± 100	0 110
Day 4 AIA								
N Rabbit	Serum	−18 h	< 0 5	25 2	1 50 ± 0 19	1065 ± 155	500 ± 222	< 0 001
AIL 1α + β	Serum	−18 h	< 0 5	12 8	1 33 ± 0 20	1153 ± 179	993 ± 199	0 174
AIL 1α	IgG	−18 h	nd	nd	nd	1051 ± 257	632 ± 283	0 023
AIL 1β	IgG	−18 h	nd	nd	nd	900 ± 178	584 ± 146	0 011
AIL 1α + β	IgC	−18 h	nd	nd	nd	1015 ± 133	981 ± 229	0 317
AIL 1α + β	Serum	6 h	nd	nd	nd	912 ± 46	1090 ± 110	0 004
AIL 1α + β	Serum	24 h	nd	nd	nd	857 ± 192	651 ± 174	0 022

Mice were injected i v with neutralizing antiserum (100 µl) or purified antibodies (0 75 mg) against mrIL 1α and mrIL 1β alone or in combination [1]Treatment was started before or after induction of AIA as indicated in the table Wash out (2 ml) of six patellae were used to determine IL 1 and IL 6 levels IL 1 and IL 6 levels of the contralateral patellae was in the range 5 10 pg/ml and 0 1 1 6 ng/ml respectively The statistical significance of differences from control (left joint) ^{35}S incorporation was calculated with Student's *t* test *p* values are indicated in the table

Table 2
Effect of anti-IL-1 pretreatment of mice on cartilage destruction in AIA

Treatment	Edema		^{35}S-PG content day 2		PG -content day 7	
	Tc-ratio Day 2	Day 7	(CPM) Left	Right	(μg/patella) Left	Right
NRS	2.4±0.3	1.5±0.3	481±108	218±57**	2.46±0.26	1.70±0.45**
AIL-1α+β	2.3±0.5	1.4±0.3	431± 95	218±70**	2.45±0.32	2.03±0.22**

Rabbit anti-IL-1 antisera (100 μl anti-IL-1α + 100 μl anti-IL-1β) or 200 μl normal rabbit serum (NRS) were given as a single i.v. injection 18 h before induction of arthritis. Edema was measured at days 2 and 7 of AIA. Proteoglycan (PG) breakdown at day 2 of arthritis was measured as *in vivo* loss of the ^{35}S-sulfate content of prelabeled patellar cartilage. Cartilage depletion of proteoglycans by arthritis was measured as loss of glycosaminoglycan content of patellar cartilage at day 7 of arthritis. Statistical significance of differences from control (left joint) was calculated with Student's *t*-test, * $p < 0.005$, ** $p < 0.0001$.

the treatment was effective. IL-6 levels in the washouts of tissue from day 1 were unchanged and only slightly decreased at day 4. This suggested that the cascade IL-1→IL-6 was not essential in this arthritis model. A recent publication demonstrated that elevations of IL-6 levels coincided with improvement of the arthritic process in RA [7]. In this sense, blockage of IL-1 and not IL-6 by anti-IL-1 antibody treatment may have a dual benefit in the management of arthritis.

Knowing that anti-IL-1 antisera pretreatment of mice effectively scavenged IL-1, we investigated how these antibodies would influence the outcome of AIA. Anti-IL-1 antisera pretreatment caused moderate suppression of joint swelling at day 4 of AIA (Table 1). The results are consistent with IL-1 as an inducer of prostaglandins known to mediate edema. On the contrary, intra-articularly injected mrIL-1 caused insignificant swelling [8].

Chondrocyte PG-synthesis was already suppressed at day 1 of AIA and carried on for days and was one of the marked events in the inflamed joint. IL-1 was a likely candidate to be involved as the IL-1 induced PG-synthesis suppression *in vivo* is well documented [8]. Pretreatment of mice with whole serum or affinity purified anti-IL-1 antibodies prevented this inhibition of PG-synthesis in AIA (Table 1). Affinity purified antibodies against an irrelevant antigen e.g. lysozyme, normal rabbit immunoglobulin or anti-IL-1 antiserum devoid of immunoglobulin had no effects. Scavenge of both IL-1α and IL-1β was necessary to normalize PG-synthesis, blockage of each separately did not effect PG-synthesis.

The suppression of chondrocyte PG-synthesis in AIA was not related to the influx of neutrophils into the joint cavity. We started anti-IL-1 treatment at 6 h after elicitating AIA and still could prevent PG-synthesis inhibition completely (Table 1). At 6 h of AIA the neutrophil influx is a prominent feature. Treatment started after the first day of arthritis, so after the peak of IL-1 production, did not affect the ongoing PG-synthesis suppression. IL-1 levels in wash-outs of inflamed tissue taken at day 4 of AIA were below the detection level of the bioassay (Table 1). We demonstrated that in this arthritis model IL-1 caused the chondrocyte synthesis suppression. Futhermore, we hypothesize that the nonresponsive state of arthritic chondrocyte to insulin-like growth factor (IFG) maintained this suppression of the PG-synthesis in this model [9].

The accelerated breakdown of ^{35}S-prelabeled PG's in AIA was not affected by the anti-IL-1 treatment (Table 2). This could be due to the modest effect of anti-IL-1 antibodies on joint inflammation. Nevertheless, anti-IL-1 antibody treatment prevented substantially the articular cartilage depletion as observed histologically and by reduced loss of glycosaminoglycans from patellar cartilage at day 7 (Table 2). We concluded that cartilage matrix depletion in AIA was caused by accelerated PG-breakdown and decreased PG-synthesis. Therefore, maintaining PG-synthesis by anti-IL-1 treatment during arthritis might be of therapeutic interest.

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CHAPTER 7

ROLE OF INTERLEUKIN-1 (IL-1), TUMOR NECROSIS FACTOR (TNF)-α AND INTERLEUKIN-6 (IL-6) IN CARTILAGE PROTEOGLYCAN METABOLISM AND DESTRUCTION: EFFECT OF IN SITU BLOCKING IN MURINE ANTIGEN- AND ZYMOSAN-INDUCED ARTHRITIS

Arthritis & Rheumatism, in press

Role of interleukin-1 (IL-1), tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) in cartilage proteoglycan metabolism and destruction: effect of in situ blocking in murine antigen- and zymosan induced arthritis.

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Objective. To determine the involvement of IL-1, TNF and IL-6 in the cartilage pathology of murine antigen-induced arthritis (AIA) and zymosan-induced arthritis (ZIA).

Methods. Arthritis was induced either by i.a. injection of zymosan in naive mice or by methylated bovine serum albumin (mBSA) in sensitized animals. Mini-osmotic pumps releasing human recombinant IL-1 receptor antagonist (IL-1ra) protein were implanted intraperitoneally two days before arthritis induction, and neutralizing antibodies directed against murine IL-1α, IL-1ß, TNFα, or IL-6 were administered one day before. Proteoglycan (PG) synthesis and degradation were assessed in patellar cartilage.

Results. Murine IL-1α and ß injected intra-articularly suppressed chondrocyte PG synthesis at doses of 0.1-100 ng. The highest dose of 100 ng TNF tested decreased the PG synthesis marginally. In contrast, the maximum dose of 1 μg IL-6 stimulated PG synthesis two days after injection. Treatment of AIA with neutralizing monoclonal antibodies against either TNFα or IL-6 neither reduced PG degradation nor the suppression of its synthesis. However, treatment with anti-IL-1 (α + ß) polyclonal antibodies totally prevented PG suppression although the initial breakdown of PG was unaffected. This effect was confirmed when IL-1ra was administered in high doses. Moreover, treatment of ZIA with anti-IL-1 (α + ß) but not with anti-TNF resulted in normal PG synthesis confirming the key role played by IL-1 in the inhibition of PG synthesis. Treatment of AIA with anti-IL-1 did not affect inflammation during the acute phase but a significant reduction in ongoing inflammation was noted at day 7 and there was a marked reduction in the loss of cartilage PG.

Conclusion. The suppression of PG synthesis in both ZIA and AIA in mice is due to the combined local action of IL-1 (α + ß) and neither IL-6 nor TNF are involved. Moreover, the normalization of PG synthesis brought about by blocking IL-1 ameliorates the cartilage damage associated with AIA.

Introduction

Rheumatoid arthritis (RA) is a systemic illness characterized by chronic inflammation of the joints and severe cartilage pathology such as joint space narrowing. Tumor-necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) are clearly involved in the arthritic process since all three cytokines are present in synovial fluid and can be detected immunohistochemically in the inflamed rheumatoid synovium (1,2). Furthermore, both local and systemic levels of each cytokine correspond with disease activity (3-5), and TNF and IL-1 have profound catabolic effects on articular cartilage explants of numerous species (6,7).

The spontaneous production of IL-1 by rheumatoid synoviocytes can be inhibited by anti-TNF-antibodies (8) suggesting that TNF was earlier in the cascade than IL-1 whereas IL-6 occupies a position later in the cascade being produced in response to either TNF or IL-1 (9,10). Furthermore, IL-1 induces IL-6 synthesis by the chondrocytes and is a cofactor in the IL-1 induced suppression of PG synthesis (11).

Intra-articular injections of TNF and IL-1 cause an influx of neutrophils into the joint and synovitis similar to that seen in experimental arthritis but only IL-1 results in marked depletion of the cartilage matrix (12-15). *In vitro*, TNF is also less potent than IL-1 in suppressing PG synthesis in cartilage explants (16,17).

Direct evidence that TNF and IL-1 play a role in the pathogenesis of experimental arthritis has been obtained in animal models in which blocking the action of these cytokines delayed the onset of collagen-induced arthritis, suppressed inflammation and ameliorated cartilage destruction which corresponded to the anti-inflammatory response (18-23). These studies focused on macroscopic scoring of the affected joints and histological evaluation but did not analyse cartilage metabolism in detail nor did they investigate the effect of anti-IL-6 treatment.

We therefore undertook to investigate the potency of IL-1, IL-6 and TNF on PG synthesis and degradation of murine cartilage and the effect of blocking endogenous IL-1, TNF or IL-6 in murine arthritis induced either by antigen or zymosan (24,25), which is a potent inducer of both IL-1 and TNF *in vivo* (26,27). The effect of treatment with neutralizing antibodies on acute joint swelling and inflammation was assessed and PG synthesis and degradation was measured in cartilage.

Materials and methods

ANIMALS

Male C57Bl/6 mice were obtained from our own breeding facilities and were given a standard diet and tapwater *ad libitum*.

CYTOKINES

Purified and biologically active mature murine recombinant IL-1α and IL-1ß were generously donated by I.G. Ottterness (Pfizer Central Research, Groton

CT, USA), purified murine recombinant IL-6 by G. Ciliberto (I.R.B.M., Rome Italy), and purified recombinant human IL-1ra by Synergen (Boulder, Colorado). Murine recombinant TNFα (carrier free) was purchased from R&D Systems Ltd (Europe).

NEUTRALIZING ANTI-CYTOKINE ANTIBODIES

Rat anti mouse TNFα monoclonal antibody (VIq) was kindly given by P.H. Krammer (German Cancer Research Center, Heidelberg, F.R.G.), rabbit anti-mouse TNF polyclonal antiserum by SL Kunkel (University of Michigan Medical School, Ann Arbor, MI, USA) and another rabbit anti-mouse TNF polyclonal antiserum by GE Grau (University of Geneva, Geneve, Switzerland). Rabbit anti mouse IL-6 polyclonal antiserum was donated by M. Fuller (Univ. Alabama, Birmingham Alabama, USA). Rat anti-mouse IL-6 monoclonal antibody was purchased by Genzyme Corp (Cambridge MA, USA). Neutralizing capacity was verified with specific bioassay for the cytokines.

GENERATION OF RABBIT ANTI-MOUSE IL-1 ANTISERUM

Polyclonal antibodies directed against each type of murine recombinant IL-1 were prepared by immunization according to the method of Hogquist *et al.* (28), with some modifications. Briefly, 250 μg IL-1 in 2.25 mL PBS was suspended in 500 μL IMJECTTH ALUM (aluminium hydroxide, Pierce, Rockford, Illinois, USA) which was injected into female New Zealand White rabbits in four s.c. injections of 500 μL. A further four s.c. injections of 500 μL CFA/PBS were given adjacent to the IL-1/ALUM injections. Every 4-6 weeks, rabbits received 50 μg IL-1 s.c. suspended in ALUM in 3 divided doses, IFA/PBS s.c. adjacent and 5 μg IL-1 i.v.. Ten days after every booster, 50 mL blood was aspirated, coagulated and serum stored at -70°C, and decomplemented at 56°C before use.

PURIFICATION AND CHARACTERIZATION OF RABBIT ANTI-IL-1 ANTIBODIES

Immunoglobulins were purified by affinity chromatographic separation on a Protein G sepharose CL4B column. Immunoglobulins (IgG) were eluted from the column with 0.1 M Glycine-HCl pH 3.0 and immediately neutralized with 50 mM Tris-HCl pH 8.0. IgG fractions were then pooled, concentrated and dialysed against PBS at ambient temperature. Antibodies directed against the α or β form of IL-1 were tested *in vitro* for their neutralizing capacity, and it was found that dilutions in the range of 1/64.000 to 1/128.000 could fully block 5 pg/mL IL-1 (lowest plateau concentration) in the NOB1-proliferation assay. Antibodies showed no neutralizing cross-reactivity against each other nor against IL-2 which was tested in the CTLL proliferation assay, IL-4 in the CT.4.S proliferation assay, IL-6 in the B9 proliferation assay, and TNFα in the L929-cytotoxicity assay. The anti-IL-1 antibodies had a half-life of more than 3 days in the circulation.

INDUCTION OF ANTIGEN-INDUCED ARTHRITIS

Mice aged between 8-10 weeks were immunized by two s.c. injections into the flank skin and two into the footpad of both forelegs, with a total of 100 μg methylated bovine serum albumin (mBSA, Sigma Chemical Company, St. Louis, Missouri, U.S.A.) suspended in 100 μL Complete Freunds Adjuvant per animal. Heat-killed *Bordetella pertussis* at $2x10^9$ organisms (National Institute of Public Health, Bilthoven, The Netherlands) were administered intraperitoneally as an additional adjuvant. Two s.c. booster injections were given with 100 μg mBSA/CFA were given in the neck region on day 7. Arthritis was induced 14 days after these injections by intra-articular (i.a.) injection of 60 μg mBSA in 6 μL saline into the right knee joint.

INDUCTION OF ZYMOSAN-INDUCED ARTHRITIS

A homogeneous suspension of 30 mg zymosan A (*Saccharomyces cerevisiae*), dissolved in 1 mL endotoxin free saline, was obtained by boiling twice, and sonic emulsification. Arthritis was induced by i.a. injection of 180 μg zymosan into the right knee.

ASSESSMENT OF JOINT SWELLING

Animals were injected s.c. with 10 μCi ^{99m}Technetium pertechnetate (^{99m}Tc) in 0.2 mL saline in the neck region. After 15 minutes, the isotope accumulates in the knee due to the increased blood flow and edema, and the ammount of ^{99m}Tc was determined by external gamma counting. Joint swelling was expressed as the ratio of the ^{99m}Tc uptake in the inflamed knee joint over its non-inflamed counterpart with a ratio higher than 1.1 indicating joint swelling.

ASSESSMENT OF PROTEOGLYCAN SYNTHESIS

Six patellae were prepared so as to minimize the amount of adjoining synovium, tendon and muscle, and placed in 2 mL RPMI-HEPES-medium (Flow Laboratories, Irvine, Scotland) supplemented with gentamicin (50 mg/L), L-glutamine (2 mM) and 40 μCi ^{35}S-sulfate. At the end of 3 hour incubation, patellae were fixed in 10% formalin, decalcified in formic acid (4%), dissected and then dissolved in 0.5 mL Lumasolve (Hicol, Oud-Beijerland, The Netherlands). The ^{35}S-content of each patella was measured by liquid scintillation counting and expressed as counts per minute (CPM).

ASSESSMENT OF PROTEOGLYCAN BREAKDOWN

Mice were injected with 50-100 μCi ^{35}S-sulfate into the peritoneal cavity 2 days before inducing arthritis. Arthritis was induced after one day and a further two days later, patellae were dissected and processed to measure the $^{35}SO_4$ content as described above.

GLYCOSAMINOGLYCAN MEASUREMENTS OF PATELLAR CARTILAGE

Patellae were fixed in ethyl alcohol (96%) and decalcified in formic acid after which the cartilage layer could be stripped from its underlying bone and subsequently digested overnight at 60°C in 200 μL of 5 mg/mL papain (Type IV, Sigma, St Louis, MO) in 0.1 M sodium-acetate pH 6.5, 10 mM L-cysteine and 50 mM disodium-EDTA per patella. The PG content per patella was estimated by the dimethylmethylene blue dye-binding at 535 nm using the colorimetric method of Farndale (29).

HISTOLOGICAL PROCESSING AND ANALYSIS OF KNEE JOINTS.

Knee joints were dissected, fixed, decalcified, dehydrated and embedded in paraffin. Standard frontal sections of 7 μm were prepared and stained with safranin-O, and counterstained with fast green. Cartilage depletion was visualized by diminished staining of the matrix and scored as 0 when normal and 1 to 3 according to the degree of cartilage depletion (loss of staining).

For autoradiographic analysis of ^{35}S-sulfate incorporation, radiolabeled sulfate was injected intraperitoneally 6 hours before dissection of the knee joints. Seven 7 μm sections of paraffin embedded joints were mounted on gelatin coated slides which were immersed in K5 emulsion (Ilford, Basildon, Essex, UK) and exposed for several weeks before being developed and stained with hematoxylin and eosin.

ANTI-CYTOKINE TREATMENT OF MICE

Antibodies were injected i.v. into the *orbita plexus* of mice, 18-24 hours before induction of arthritis. Each experimental group consisted out of at least 7 animals.

ANTI-IL-1 (α + β) ANTIBODY TREATMENT

Mice were injected i.v. with 200 μL standard dose of 2 mg purified rabbit anti-IL-1 antibodies with a total neutralizing capacity of 32 ng of both subtypes of IL-1 when tested in the NOB1-assay, and was sufficient to block the effect of 1 ng of IL-1α and ß on PG synthesis completely *in vivo*. Normal rabbit IgG's or polyclonal anti-ovalbumin antibodies, served to control the non-specific effects of treatment.

ANTI-TNF AND ANTI-IL-6 TREATMENT

Mice were given a dose of 90.000 U neutralizing monoclonal antibodies directed against TNFα (V1q) which was sufficient to block 7.8 μg of TNFα in the L929 bioassay. Some mice were given rat anti-mouse IL-6 IgG1 with a total neutralizing capacity of 175 ng IL-6 in the B9-bioassay whereas others received normal rat IgG as a control.

TREATMENT OF MICE WITH IL-1ra

Mini-osmotic pumps (Alzet 1007D, Alza corp., Palo Alto CA, USA) were

implanted into the peritoneal cavity two days before arthritis induction, and set to release 37.5 μg of IL-1ra per hour for the next 7 days. The mean steady-state level of IL-1ra in blood was 4.7 μg/mL from the first day after implantation as was measured in the NOB1-assay.

Results

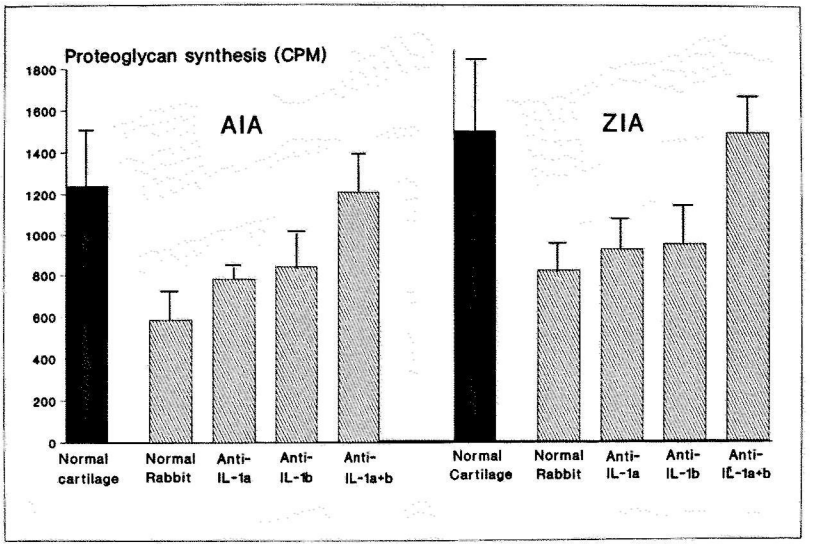
Effect of IL-1, TNF and IL-6 on PG metabolism in vivo.
Intra-articular injection of IL-1α or ß suppressed PG synthesis in patellar cartilage within a day of administration and was dose-related (Table 1). Marked suppression of 50-60% lasted at least two days and recovered thereafter.[13] Chondrocyte PG synthesis was only suppressed by 100 ng of TNFα, and lower doses having no effect after one day but actually stimulating PG synthesis after two days. Low doses of IL-6 also had no effect whereas 1 μg significantly enhanced the PG synthesis by 32% after two days.

Table 1: In vivo effect of IL-1, TNF or IL-6 on PG synthesis.

Dose	*PROTEOGLYCAN SYNTHESIS (% of Normal Cartilage)*[f]					
ng/ joint	*IL-1α Day 1*	*IL-1β Day 1*	*TNFα Day 1*	*TNFα Day 2*	*IL-6 Day 1*	*IL-6 Day 2*
0.1	70 (12)*	90 (17)	nd	nd	nd	nd
0.3	65 (17)*	72 (14)*	nd	nd	nd	nd
1	50 (7)**	54 (13)**	97 (37)	148 (23)	81 (10)	94 (21)
10	43 (9)**	48 (15)**	91 (29)	113 (19)	105 (26)	105 (33)
100	47 (9)**	44 (7)**	80 (17)*	79 (29)	103 (23)	120 (22)
1000	nd	nd	nd	nd	104 (24)	132 (23)*

Murine recombinant IL-1, TNF or IL-6 were injected into the right knee joint cavity.
[f] PG synthesis was determined by $^{35}SO_4$ incorporation *ex vivo* and expressed as a percentage (SD) of the normal synthesis in patellae of joints injected with saline at day 1 and 2 after injection.
Significantly different (*, $p<0.05$; **, $p<0.01$) compared to the saline injected joint (Mann Whitney-U-test of CPM-values).

Effect of anti-cytokine pre-treatment on PG synthesis in murine antigen- and zymosan-induced arthritis.
Chrondrocyte PG synthesis was markedly suppressed in both murine arthritis models. Anti-IL-6 and anti-TNF antibody pre-treatment of mice did not reverse the inhibition of PG synthesis at day 2 of AIA and ZIA
whereas pre-treatment with anti-IL-1 (α+β) did (Figure 1A). Selective elimination of either IL-1α or IL-1β did not prevent the suppression of PG synthesis (Figure 1B), indicating that both subtypes reached optimal effective



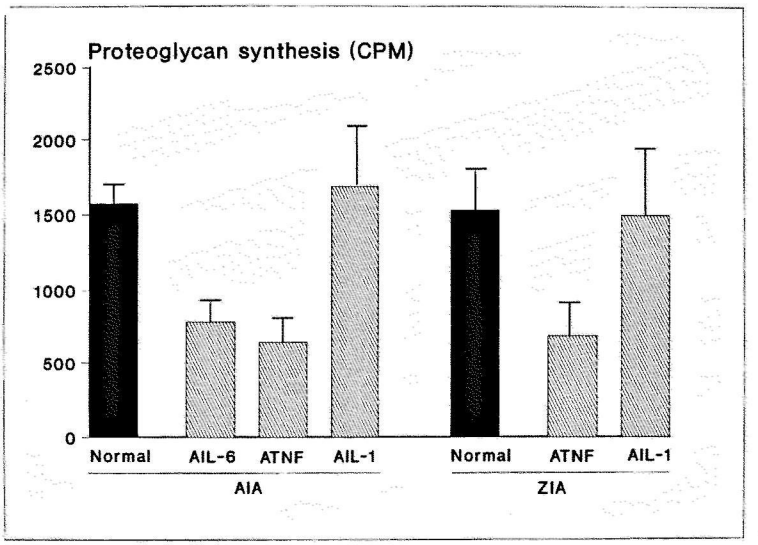


Figure 1: Role of cytokines in PG synthesis suppression of antigen-induced arthritis (AIA) or zymosan-induced arthritis (ZIA). PG synthesis was assessed in patellae at day 2 of arthritis by $^{35}SO_4$ incorporation ex vivo. A) Mice (n=7 per group) received rat anti-mouse IL-6 monoclonal antibodies (AIL-6) [total neutralizing capacity of 175 ng of IL-6 in the B9-bioassay], or rabbit anti-mouse IL-1 polyclonal antibodies (AIL-1) [neutralizing capacity of 32 ng of both IL-1 subtypes in the NOB1-bioassay, a dose found to be effective in blocking the effect of 1 ng of intra-articularly injected IL-1 on PG synthesis], or rat anti-mouse TNF monoclonal antibody (ATNF) [with a neutralizing capacity of 90.000 U of TNFα in the L929-bioassay] intravenously, one day before arthritis induction. B) Relative role of IL-1α and IL-1β in the suppression of PG-synthesis in both arthritis models. Mice received rabbit anti-IL-1 polyclonal antibodies against either IL-1α and IL-1β separately or against both subtypes intravenously, one day before arthritis induction. Mice receiving 1 mg of normal rabbit IgG served as a control.

concentrations in the joints. The acute joint swelling in AIA was as severe as in ZIA on the second day of arthritis. In AIA, joint swelling was not significantly affected by treatment with anti-IL-1, anti-TNF or anti-IL-6, and in ZIA it was only moderately reduced by 18% by anti-TNF but not by anti-IL-1. Effect of IL-1ra on PG synthesis in AIA.

In the first experiments, mice received bolus injections of either 2 mg/kg or 10 mg/kg IL-1ra into the peritoneal cavity, before and during arthritis and PG synthesis remained significantly suppressed at day 2 of arthritis (Table 2). In further experiments, the high amount of IL-1ra delivered to mice via mini-osmotic pumps prevented the inhibition of the PG synthesis at day 2 of AIA and confirmed the results obtained after pre-treatment with anti-IL-1 antibody in AIA.

Consequence of anti-IL-1 pre-treatment on cartilage pathology in chronic phase of AIA.

In AIA, PG synthesis was suppressed for the first 7 days of arthritis or more whereas the suppression in ZIA was more transient. A single intravenous injection of anti-IL-1 antibodies prior to arthritis induction, fully prevented the suppression of PG synthesis for this whole period (not shown). Autoradiography of ^{35}S pulse labeled knee joints at day 4 of AIA demonstrated marked inhibition of label incorporation indicating reduced PG synthesis in the cartilage matrices. Normalized incorporation was noted after anti-IL-1 pre-treatment of the arthritic mice (Figure 2).

Table 2: Effect of IL-1 receptor antagonist (IL-1ra) protein on PG synthesis in AIA.

	PROTEOGLYCAN SYNTHESIS‡					
	Experiment I *2 mg/kg IL-1ra*⌊		*Experiment II* *10 mg/kg IL-1ra*+		*Experiment III* *37.5 μg/h IL-1ra* ⊢	
Treatment	*Normal*	*AIA*	*Normal*	*AIA*	*Normal*	*AIA*
Saline	1172 (101)	431 (69)	nd	nd	1270 (139)	500 (153)
IL-1ra	1107 (141)	598 (142)	1230 (140)	627 (93)	1241 (251)	1268 (228)

‡ $^{35}SO_4$ incorporation in patellae *ex vivo* 48 hours after induction of AIA, expressed as the mean (SD) in at least 6 patellae.

+ IL-1ra (2 mg/kg) or saline was injected into the peritoneal cavity two hours before arthritis induction and at 4, 10, 16, 22, 28, 46 hours after arthritis induction. Total cumulative dose is approximately 0.35 mg/mouse.

⌊ IL-1ra (10 mg/kg) or saline was injected into the peritoneal cavity two hours before arthritis induction and every 3 hours the first day, and every 6 hours the second day of arthritis. Total cumulative dose is approximately 3 mg/mouse.

⊢ Mini osmotic pumps were implanted i.p. two days before arthritis induction and set to deliver 37.5 μg IL-1ra per hour or saline (control group) for the next 4 days. Total cumulative dose is approximately 3.6 mg/mouse. Representative of three experiments.

** Significantly different $p<0.01$, compared to the saline treated arthritic joint (Mann Whitney-U-test).

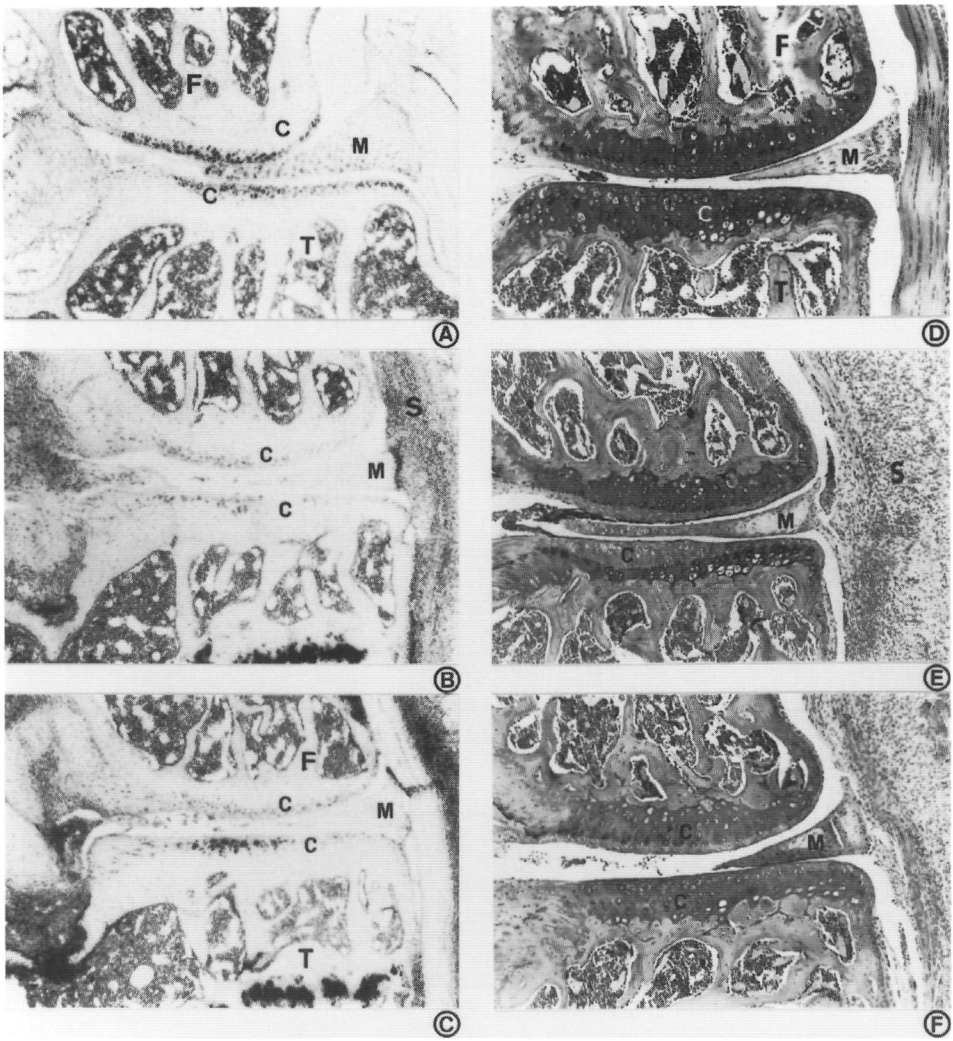


Figure 2. Histology of the femoral-tibial joint of the knee. Photographs (A, B, C) represents autoradiography of $^{35}SO_4$-incorporation into newly synthesized PGs. Photographs (D, E, F) represents safranin-O stained sections. Diminished staining indicating cartilage PG loss. A and D: normal contralateral joint. B and E: arthritic joint day 4 AIA. C and F: arthritic joint of anti-IL-1 treated mouse. C: cartilage. F: Femur. T: Tibia. M: Meniscus.

In the next experiment, cartilage degradation was accelerated since the ^{35}S-sulfate content in prelabeled patellar cartilage at day two was reduced by 52 ± 10% in AIA and 43 ± 11% in ZIA. Anti-IL-1 (α + β) pre-treatment did not prevent this in either form of arthritis with losses of 50 ± 12% and 35 ± 9% being measured, suggesting that IL-1 was not involved in initiating this process.

The combined action of reduced synthesis and enhanced degradation led to a profound loss of cartilage PG in AIA, which was much less by pre-treatment with anti-IL-1, as reflected by PG measurements of cartilage at days 4 and 7 of AIA (Table 3). Histological analysis of whole knee joints showed significant amelioration of the damage to the cartilage. Safranin-O staining of the articular cartilage matrices was greater in the arthritic joints of anti-IL-1 treated as compared to control treated mice indicating a higher PG content (Figure 2).

Although blocking of IL-1 had no anti-inflammatory effect on the acute inflammation, ongoing inflammation of the joint was much lower in later phases of AIA after pre-treatment with anti-IL-1. Joint swelling and synovitis were significantly reduced at day 7 of AIA (Table 3).

Table 3. Effect of anti-IL-1 treatment on inflammation and cartilage loss in AIA.

Treatment[J]	*Joint swelling*[ʃ] *(Tc-ratio)*	*Synovitis*[+]	*Glycosaminoglycan content*[+] *(µg per patella)*	
	Day 7 AIA	*Day 7 AIA*	*Day 4 AIA*	*Day 7 AIA*
N.Rabbit IgG	1.44 (0.25)	2.3 (0.8)	1.56 (0.50)	1.70 (0.45)
Anti-IL-1	1.23 (0.16)*	1.3 (0.9)*	2.30 (0.46)**	2.03 (0.22)

J Normal rabbit IgG or antibodies against IL-1 (α + ß) were injected intravenously one day before arthritis induction.

ʃ Joint swelling was expressed as a ratio (SD) of the enhanced uptake of ^{99m}Technetium pertechnetate in the arthritic over the contralateral normal joint (M&M).

╫ Whole knee joint sections were analyzed for leukocyte infiltration into the synovium and arbitrary scored from 0; no cells, to a maximum score of 3; large number of cells.

\+ Glycosaminoglycan content measured as described in M&M. Content in patellar cartilage of the contralateral knee joint was 2.58 ± 0.39; day 4, 2.45 ± 0.31 µg; day 7.
Significantly different compared to the normal rabbit treated animals, *; $p < 0.05$, **; $p < 0.01$, ns; not significant from normal rabbit treated animals (Mann Whitney-U-test).

Discussion

Direct evidence for the involvement of cytokines in the process of joint inflammation and cartilage destruction in RA is still lacking. We therefore undertook to investigate in a comparative study the potency of TNFα, IL-1 and IL-6 to affect chondrocyte function *in vivo* and the effect of selective blocking on murine arthritis.

The PG synthesis was markedly suppressed after i.a. injections of IL-1α or IL-1β whereas higher doses of TNFα were needed for this. In previous studies, we and others convincingly demonstrated that i.a. injections of IL-1 into synovial joints causes marked cartilage PG degradation and cartilage depletion in various species (12-14). Of interest, this effect seems to be independent of the inflammatory reaction (13,30). A single i.a. injection of TNFα did not cause edema nor enhanced PG degradation in the tested dose-range of 1-100 ng in mouse (data not shown). Although TNF may cause cartilage destruction *in vitro* (16), evidence for such a role *in vivo* is lacking (14,15).

TNF and IL-1 are potent inducers of IL-6 production but we clearly showed that IL-6 is not a destructive mediator in the murine joint and even may play a protective role in the joint since IL-6 stimulates PG synthesis. Moreover, IL-6 enhances the expression of inhibitors (e.g. TIMP) of cartilage destructive enzymes in synovial fibroblasts (31).

It was shown in rheumatoid synovia that TNF may be an important driving force in the production of IL-1 (8). Although TNF does not seem to be a major cartilage destructive mediator, its regulating role makes it an interesting target for therapy. Recent studies with chimeric antibodies to TNFα proved efficacious in suppressing signs of inflammation in short trials (32), but the protection against ongoing cartilage destruction has yet to be demonstrated. Given the existence of separate pathways of IL-1 production, in addition to TNF driven pathways, it is tempting to suggest that blocking of IL-1 would be a more valid approach with respect to amelioration of cartilage destruction.

Pre-treatment of mice with rabbit polyclonal anti-IL-1 antibodies protected the chondrocyte function in both ZIA and AIA. The IL-1 subtypes had to be blocked together to prevent suppression of PG synthesis in arthritis whereas pre-treatment with anti-TNF or anti-IL-6 antibodies had no effect. This result is in agreement with the relative potencies of these cytokines after i.a. injection into the murine knee joint. Although it is reported that IL-6 is a cofactor in IL-1 induced suppression of PG synthesis in human cartilage explants (11), this has yet to be confirmed *in vivo*.

A major role of TNF and/or IL-1 in cartilage PG degradation can not be deducted from the present study in the acute phase of murine AIA and ZIA, and this result is in line with observations in AIA in the rabbit (33,34). This may point to mere overkill by other mediators, or imply that IL-1 levels in AIA are anyway insufficient. In that respect it is intriguing to note that chondrocyte PG synthesis inhibition can be achieved with relatively small doses of IL-1, whereas considerable IL-1 levels are needed to cause substantial PG degradation (13,16,17).

The overall effect of cytokine neutralization seems to depend both on the type and the phase of the arthritis. Anti-TNF treatment in collagen-induced arthritis (CIA) resulted in a marked amelioration of joint inflammation and cartilage destruction in a prophylactic protocol, but was

markedly less efficient when treatment was started in the established disease (36). In contrast, neutralization of IL-1 was still efficacious when treatment was started late and marked protection of cartilage damage was observed (19,35). In the present study, anti-TNF pre-treatment using the same antibodies (V1q) as was used in CIA, did not diminish acute joint swelling nor did it protect against cartilage damage in murine AIA. Furthermore, neutralization of IL-1 also using the same anti-IL-1 antibodies as in CIA did not reduce acute joint inflammation either, yet markedly ameliorated cartilage destruction after day 2 of AIA.

These findings in the various models suggest that protection against cartilage destruction with neutralizing anti-cytokine antibodies can be obtained in two ways. First, indirect effect, when IL-1 and/or TNF are key elements in the inflammatory process. Second, a direct effect, when inflammation is caused by substantial overkill by other mediators, yet elimination of a destructive mediator like IL-1 may still cause substantial protection. In the first situation a direct, destructive role of IL-1 can never be proven, but in the present study in AIA and ZIA, where the acute inflammation seems highly IL-1 and TNF independent, a key role of IL-1 in the suppression of PG synthesis is now established. However, we cannot exclude that the cartilage amelioration in the anti-IL-1 treated mice was due to decreased PG degradation either directly or as a consequence of relief of the ongoing flammation as was shown at day 4 and 7 of AIA.

The role of IL-1 in PG synthesis suppression is confirmed in IL-1ra treated mouse. Conflicting results were reported in the past. Lewthwaite et al. (34) could block IL-1 induced inflammation in the rabbit, but were unable to protect PG synthesis in rabbit AIA using repeated s.c. injections with huIL-1ra. Whooley et al. (18) reported also a failure to modulate murine AIA. We had similar, negative observations using repeated IL-1ra injections, but efficacy was clearly proven when continuous high levels were generated using Alzet mini-osmotic pumps. Using a similar approach we recently proved efficacy also in murine CIA and ICA (manuscripts in preparation). The relevance of this approach is obvious, given the poor pharmacokinetic profile of IL-1ra. Moreover, continuous blocking of almost all IL-1 receptors is needed to prevent cell activation, obliging a continuous excess of at least 1000-10.000 fold amount of IL-1ra (36).

In RA much emphasis is now focused on blocking TNFα using either chimeric antibodies or fusion proteins with TNF soluble receptor. In addition, first trials are underway with IL-1ra. Our experimental studies suggest that IL-1 is an important target in protection against cartilage destruction. Moreover, continuous high levels of IL-1ra are needed to control IL-1 in arthritic processes and it is debatable whether IL-1ra dosages used in clinical trials are high enough. It is hoped that antagonist proteins with a beter profile, or selective inhibitors of IL-1 production become available in the near future.

Our present study does not show efficacy of anti-IL-6 antibody treatment. Given the high levels of IL-6 in the circulation and inflamed joints, it can not be excluded that some IL-6 escapes neutralization. This holds in particular for the local production in cartilage, since antibodies will not penetrate to a great extent. The role of IL-6 remains to be elucidated but we expect that it plays a protective role in arthritis.

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CHAPTER 8

FLARE OF EXPERIMENTAL ARTHRITIS IN MICE WITH MURINE RECOMBINANT IL-1

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Flare-up of experimental arthritis in mice with murine recombinant IL-1

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SUMMARY

Intra-articular injections of murine recombinant IL-1 (mrIL-1) during the chronic phase of antigen-induced arthritis (AIA) induced a flare-up of the smouldering inflammation The exacerbation was characterized by acute and transient joint swelling and this coincided with the extravascular accumulation of neutrophils IL-1 injected into arthritic joints of neutropenic mice demonstrated that joint swelling was independent of the neutrophil influx into the joint Both phenomena were absent when IL-1 was injected into a naive joint The IL-1-induced flare-up was not T cell mediated as in the antigen-induced flare-up, and suggestive evidence is presented that IL-1 sensitivity depended on the resident macrophage population This explained why the hypersensitivity is not restricted to the immunologically mediated arthritis but reflects a more general hypersensitivity of previously injured joints e g zymosan-induced arthritis and IL-1-affected joints In addition IL-1 could also potentiate the antigen-specific flare-up of chronic AIA and prolongs the duration of the exacerbation Our data indicate that joints bearing a chronic infiltrate are at risk from exacerbations in two ways a T cell mediated rechallenge with antigen and a non-specific reactivation by systemic and local IL-1 generation

Keywords IL-1 synovial joint flare-up experimental arthritis mice

INTRODUCTION

The clinical course of rheumatoid arthritis (RA) is characterized by pathology of the connective tissues and recurrent secondary flare-ups of the smouldering chronic inflammation IL-1 has been reported to be present in synovial fluid of patients with RA [1-3] Noteworthy is the linkage of the spontaneous IL-1 synthesis by human monocytes to the early onset of the relapse of RA [4] Furthermore, plasma IL 1β levels correlated with patients' disease activity and followed secondary flare ups in RA [5]

Effects of IL-1 on single cell populations and tissue explants taken from the joint strongly imply that IL 1 is involved in the arthritic process [6-8] Furthermore *in vivo* administration of IL-1 in rodents illustrated that IL-1 had cartilage destructive and inflammatory activities [9-12] affecting the synovial joint comparable to experimental models of arthritis We recently demonstrated that IL-1 is directly involved in the pathological processes of arthritis Antigen-induced arthritic mice treated with neutralizing antibodies raised against IL-1 demonstrated that chondrocyte synthesis inhibition and to some extent cartilage depletion during arthritis were caused by *de novo* synthesized IL-1 (unpublished observations)

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IL-1 is not only responsible for most of the pathology of connective tissues in the synovial joint but could also modulate the inflammatory process of arthritis Several reports demonstrated that IL-1 also had an immunoregulatory role in several animal models of arthritis In collagen-induced arthritis in DBA mice and in the spontaneous arthritis in MRL mice IL-1 treatment increased incidence and enhanced onset of arthritis [13 15] In rats pre- and post-treatment with IL-1 locally reduced joint swelling and histopathology of antigen-induced arthritis [16] In peptidoglycan-polysaccharide-induced arthritis in rat ankles IL 1 induced exacerbations of arthritis but clear-cut clinical and histological differences were only found after repeated IL-1 injections into those previously injured joints [17] Overall exogenous IL-1 had intricate effects on pathogenesis of experimental arthritis and this may depend on route site (predisposed) and time of administration

The antigenic reactivation of the subsided antigen induced arthritis (AIA) in rabbits and mice closely resembles the relapse in RA acute onset of the flare-up reaction marked joint swelling and abundant neutrophil influx are evident [18 19] The flare-up of AIA is T cell dependent [20] and it seems obvious that IL-1 played an important role in the flare-up because of its well known T cell activating properties [21]

In this study we compared the effect of IL-1 in various stages of both immune and non-immune arthritis IL-1 caused a relapse of the smouldering joint inflammation in the chronic

phase of arthritis Joint swelling and marked neutrophil influx were evident The kinetics, mechanism and prerequisites of the flare-up were studied in more detail It was demonstrated that previously injured joints bearing a macrophage-rich infiltrate displayed clear-cut IL-1 hypersensitivity

MATERIALS AND METHODS

Chemicals

Murine recombinant IL-1α [22], and IL-1β were expressed in *Escherichia coli* and the purified mature IL-1 was biologically active Biological activity was verified in the LAF assay and IL-1 was found to give 1 unit activity (proliferation 1/2 maximal) consistently in the 10 40 pg/ml range Screening for endotoxin activity (Limulus assay) was negative up to a concentration of 100 μg ml

Nitrogen-mustard (Mustine-hydrochloride) was obtained from The Boots Company (Nottingham, UK), methylated bovine serum albumin (mBSA), concanavalin A (Con A), and zymosan A (*Saccharomyces cerevisiae*) were obtained from Sigma Chemical Co (St Louis, MI)

Immunization and induction of arthritis

Male C57Bl 6 mice 6 8 weeks of age were immunized with 100 μg mBSA in FCA and *Bordetella pertussis* as an additional adjuvant as described before [19] Monoarthritis was induced 21 days after the start of the immunization by injecting 60 μg mBSA in 6 μl phosphate-buffered saline (PBS) in the left knee joint

The non-immune arthritis was induced by intra-articular injection of 1 8 180 μg zymosan Zymosan (30 mg) was dissolved in 1 ml saline through heating up to 100 C twice and sonificated afterwards to obtain a homogeneous suspension

Induction of flare-up

The flare-up reaction was induced 7–35 days after onset of arthritis by local or systemic injection of IL-1, mBSA or both

Treatment of experimental arthritis

Lymphocyte depletion Goat anti-mouse lymphocyte serum (ALS), titre 1 6400, showed a 98% lysis of murine splenic lymphocytes in a cytotoxicity test Mice were injected with 0 5 ml ALS intraperitoneally 72, 48 and 24 h before flare-up induction The control group received the same amount of heat-inactivated normal goat serum intraperitoneally As a control, delayed-type hypersensitivity (DTH) reaction (24 h) against mBSA (5 μg) in the ear was completely prevented by ALS treatment

Neutrophil depletion Nitrogen-mustard, 1 mg/kg, was administered orally once a day for four consecutive days or 2 days intravenously 2 mg/kg before IL-1 administration After these regimens, the number of neutrophils in the blood was decreased by 90% The DTH-reaction against mBSA (5 μg) was approximately 50% diminished by these nitrogen mustard treatments, indicating that an inflammatory reaction was still possible in these mice

^{99m}Tc uptake measurements

Joint inflammation was determined by measurements of ^{99m}Tc pertechnetate uptake in the knee joint [23,24] Briefly, approximately 10 μCi ^{99m}Tc in 0 2 ml saline were injected subcutaneously in the neck region of mice After 10 min, animals were sedated by intraperitoneal administration of 4 5% (w w) chloral hydrate, 0 1 ml 10 mg of body weight, and the accumulation of the isotope in the knee due to increased blood flow and tissue swelling was determined by external gamma counting The severity of inflammation was expressed as the ratio of the ^{99m}Tc uptake in the affected (left) over the contralateral control (right) knee joint

IL-1 retention measurements

IL-1 was radioiodinated using Bolton and Hunter reagent (Amersham, UK) according to the manufacturer's instructions [^{125}I]labelled IL-1 was injected into the right knee joint, whereas the left knee joint received saline At various hourly time points thereafter, the [^{125}I]radioactivity of both knees was measured by external gamma counting Readings for the right knee were corrected by subtraction of background activity in the left knee Retention was expressed as a percentage of the initial count rate measured immediately after [^{125}I]IL-1 injection Subsequently quantitative measurements were made on dissected patellae

Histology of knee joints

For histological scoring of the inflamed knees, the left and right joints were dissected and processed for histology as previously described [19] Standard frontal sections (7 μm) were prepared and stained with haematoxylin and eosin The arthritic score was determined by grading the infiltrate and the exudate, the extent was graded 0–3 where 0 = no change and 3 = large cell numbers

RESULTS

IL-1-induced joint swelling in the chronic phase of antigen-induced arthritis

Intra-articular injection of mrIL-1α into a previously inflamed joint (week 4) caused a dose-dependent joint swelling oedema and plasma leakage into the joint space (Fig 1a) The IL-1 hypersensitivity existed for both types, IL-1α and IL-β (Fig 1b) Heat-inactivated IL-1 was without effect The arthritic joint also responded to circulating IL-1, although high doses must be applied (Fig 1b) A naive normal joint was less sensitive for IL-1 and lacked a significant joint swelling response after injection The onset of IL-1 induced joint swelling in arthritic joints was rapid, a peak was found at 6 h, and a decline to baseline had already occurred at 24 h (Fig 2)

The hypersensitivity of arthritic knees was not due to an altered clearance of IL-1 The retention of iodinated IL-1, still fully biologically active, was similar in a naive and an arthritic joint, $T_{1/2}$ = 45 min (data not shown)

Histology of IL-1-induced flare-up

Histological sections taken from arthritic joints after IL-1 injection demonstrated that joint swelling coincided with a marked influx of polymorphonuclear (PMN) cells, predominantly neutrophils, into the joint (Fig 2) By contrast, in week 2 of AIA the background inflammation consisted of a synovial infiltrate of mononuclear cells Although less distinct, high doses of IL-1 also elicited a granulocyte influx into a naive joint, but without joint swelling

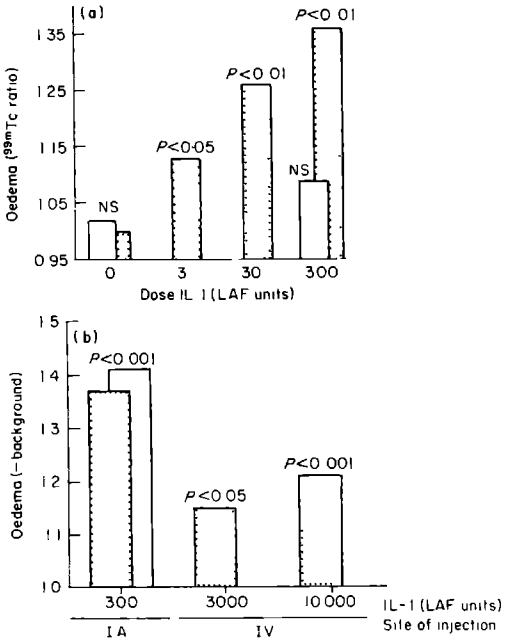

Fig. 1. IL-1-induced joint swelling (oedema) in 4-week old antigen-induced smouldering joint inflammation (a) Dose response of intra-articularly injected IL-1 in arthritic compared to naive joints □, control joint ■ arthritic joint (b) Comparison of flare-up induced by intra articular injections of IL-1α and IL-1β, and systemic IL-1β ○ Statistical analysis of ^{99m}Tc uptake as described in Table 1

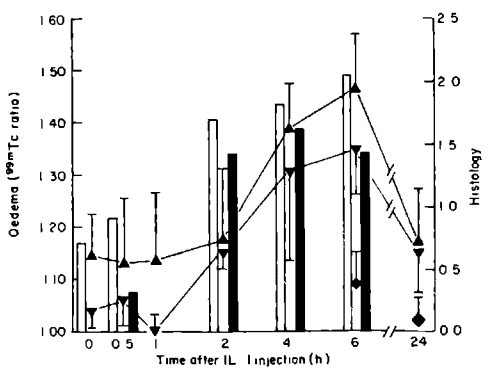

Fig. 2. Kinetic of flare-up of arthritis IL-1 (300 U) was injected into 2 week-old zymosan- (▲) or mBSA- (▼) induced arthritis At different time points after IL-1 injection the ^{99m}Tc uptake was measured in separate groups of mice As a control, saline was injected into antigen induced arthritic joints (◆) Histology of AIA showed both synovial infiltration (□) and exudation into the jointspace (■) of neutrophils

Table 1. IL-1-induced flare-up of experimental arthritis is independent of neutrophil accumulation and *de novo* prostaglandin synthesis

	Oedema (^{99m}Tc ratio)*		Exudate	
Pre-treatment	Saline	IL-1	Saline	IL-1
Nitrogen mustard				
–	1 14±0 08	1 36±0 10†	0 2±0 3	2 1±0 5
+	1 15±0 04	1 32±0 03‡	0 2±0 3	0 4±0 4
Indomethacin				
–	1 03+0 03	1 62±0 21§	0 4±0 4	2 9±0 2
+	ND	1 34±0 27	ND	2 1±0 5

Nitrogen mustard (2 mg/kg) was injected intravenously (into orbital plexus) at day −3, and −1, before induction of IL-1 flare, and caused >90% depletion of circulating neutrophils Indomethacin (2 mg kg) (solubilized in methyl cellulose) was given orally for three successive days (3, minus (×3) −2, and −1), before the IL-1 injection *Oedema (joint swelling) was expressed as ratio of ^{99m}Tc uptake by inflamed joint over the contralateral (non-inflamed) joint A value near 1 0 is typical for non-inflamed joints Statistical analysis of ^{99m}Tc ratios of IL-1-injected joint compared with saline-injected joints was done by the two tailed Wilcoxon matched pairs signed rank test †$P<0\,05$, ‡$P<0\,001$, §$P<0\,001$ Representive experiment carried out in week two of AIA, and identical results were obtained in zymosan-induced arthritis ND Not done

Characteristics of the IL-1 flare-up

PMN cell influx and joint swelling are independent phenomena, as demonstrated by the IL-1-induced joint swelling in neutropenic arthritic mice (Table 1) This indicates that oedema formation is not dependent on the IL-1-induced PMN cell extravasation

In general, joint swelling can be effectively suppressed by preventing prostaglandin synthesis [25] Mice were treated with indomethacin (2 mg/kg per day, orally) for 3 successive days before injection of IL-1 into the smouldering arthritis Apparently, the IL-1-induced joint swelling was only partly mediated, and the extravascular neutrophil accumulation not mediated, by prostaglandin (Table 1)

Reactivation of various types of joint inflammation with IL-1

Antigen-induced exacerbation of the smouldering arthritis (AIA) is known to be T cell mediated We now investigated whether the IL-1 flare-up was also T cell dependent Animals were pre-treated for 3 successive days with ALS This did not affect the subsequent joint swelling after IL-1 injection, and had only a minor effect on the neutrophil influx (Table 2) T cell independence of the IL-1 hypersensitivity was further emphasized by the reactivation of the non-immunologically mediated zymosan-induced arthritis (ZIA) by IL-1 (Fig 2)

ZIA and AIA differ in their chronicity ZIA remits within 2–3 weeks whereas in AIA the smouldering inflammation is maintained for 4–5 weeks The subacute phases of ZIA and AIA were equally sensitive to IL-1, but IL-1 hypersensitivity lasted longer in AIA than in ZIA Overall, the hypersensitivity for IL-1 followed the arthritic course (Fig 3a)

The above data indicate that IL-1-induced flare-up reflects a general hypersensitivity in previously injured joints In addition, we injected IL-1 into a joint previously injured with IL-1

Table 2 IL 1 induced exacerbation is not T cell mediated but depends on resident inflammatory cells

Pre treatment	Challenge: Substance	Challenge: Route	Oedema ^{99m}Tc ratio	Histology exudate
Saline	Saline	IA	1 18±0 12	0 9±0 5
Nitrogen mustard*	Saline	IA	1 01+0 10	0 1±0 2
Saline	IL 1	IA	1 51±0 10	2 2±1 2
Nitrogen mustard	IL 1	IA	0 98+0 28‡	0 1±0 2
Goat serum	IL 1	IA	1 56+0 11	2 3±0 6
ALS†	IL 1	IA	1 48+0 14	1 2±1 0
Goat serum	mBSA	IV	1 38+0 17	1 8±1 1
ALS	mBSA	IV	1 17±0 08§	0 1±0 3§

* Nitrogen mustard (1 mg kg) was given daily for 4 successive days and 2 days later flare up was induced †Arthritic mice were pre treated with 0 5 ml anti lymphocyte serum (ALS) intraperitoneally at days −1 −2 and −3 before antigen (mBSA) or IL 1 challenge This completely abrogated the antigen induced delayed type hypersensitivity (DTH) reaction in the ear mBSA (300 μg) was injected intravenously and the exacerbation was measured 24 h later IL 1 (300 U) was intra articularly injected and the exacerbation was measured 6 h later ‡ Statistical analysis of ^{99m}Tc ratios of treated (nitrogen mustard or ALS) mice compared with the control (saline or normal goat serum) treated mice with the same (saline IL 1 or mBSA) challenge Method and statistics as described in legend of Table 1

Significant joint swelling (^{99m}Tc ratio 1 27±0 10 *versus* 1 02±0 02 background value) was found 6 h after the third of a series of IL 1 injections given on alternate days Rechallenge with IL 1 1 week later did not induce oedema (^{99m}Tc ratio 1 02±0 08) indicating that this state of hypersensitivity was short lived

Prerequisites for IL 1 hypersensitivity

IL 1 hypersensitivity depended on severity of primary arthritis Various doses of zymosan were injected into the knee joints and IL 1 induced flare ups were determined 2 weeks later Zymosan induced a dose-dependent joint inflammation in the range of 6 180 μg as measured with ^{99m}Tc uptake at day 3 (Fig 3b) There was almost complete remission of the inflammation 2 weeks later at which time marked flare ups produced by IL 1 were only seen in the joints previously injured with the highest zymosan doses

Total depletion of the cellular infiltrate by prolonged nitrogen mustard treatment resulted in the complete abrogation of the IL 1 induced flare up (Table 2) This indicates that the infiltrate is involved in the IL 1 induced flare up and probably not a mere change in the endothelium

To investigate this further Con A was injected into naive knees to induce a mononuclear cellular (MNC) infiltrate [26] with little joint swelling After only 2 days the joint swelling had vanished but a clear MNC infiltrate remained This kind of infiltrate renders the knee joint susceptible to IL-1 induced oedema and suggest that macrophages mediate the IL 1 flare up (Table 3)

Additive effect of IL 1 on antigen induced flare up of AIA

Chronic AIA could be reactivated by tiny amounts of antigen dependent on the state of T cell mediated local hypersensitivity

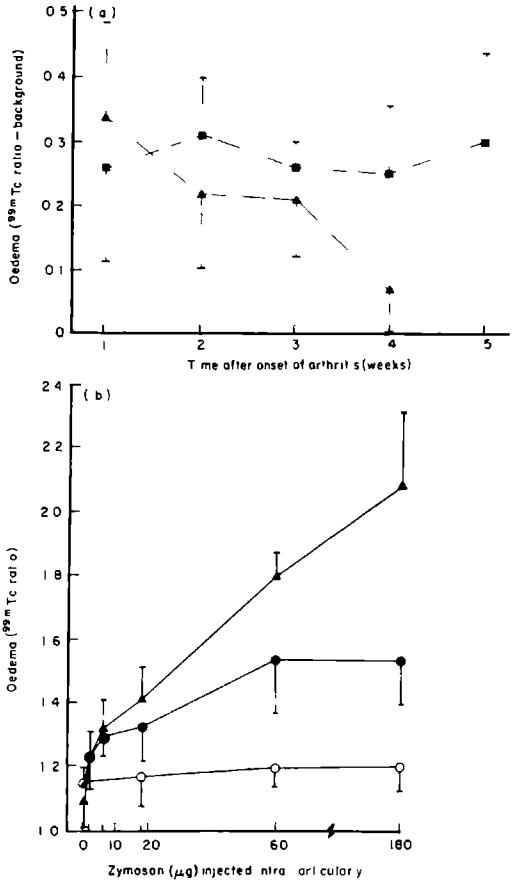

Fig 3 Exacerbation of arthritis depended on course of the joint inflammation (a) and extent of primary joint inflammation (b) (a) Induction of flare up at different time points after onset of zymosan induced arthritis (ZIA) (180 μg zymosan) (▲) or antigen induced arthritis (AIA) (60 μg mBSA) (■) with intra articularly injected IL 1 (300 U) Oedema formation was measured 6 h later and background inflammation was subtracted from the IL 1 induced flare up (b) Various concentrations of the arthritogen zymosan were injected into the knee joints After 3 days the primary joint inflammation was measured with ^{99m}Tc uptake (▲) Two weeks later IL 1 was injected into the subsided arthritis (●) and joint swelling was measured after 6 h as a control saline was injected into the arthritic joints (○)

One hundred nanograms are always sufficient but 10 ng gives variable exacerbations We investigated whether IL 1 could synergize with the antigen induced flare up Figure 4 shows that mBSA caused a small but insignificant joint swelling The combination of mBSA and IL 1 resulted in a profound flare up which was higher than that found with antigen or IL 1 alone Moreover the flare up was more protracted (Fig 4) The same additive effect of IL-1 was also found in the antigenic reactivation of the Streptococcal cell wall induced arthritis in mice (data not shown) In contrast, severity of primary inflammation was

Table 3. IL-1-induced exacerbation of Con A-affected knee joints

Time (h)	Injection at 42 h	Oedema, ^{99m}Tc ratio	Histology		Dominant cell type
			Exudate	Infiltrate	
6	—	1·22±0·11	0·7±0·3	1·2±0·6	PMN
24	—	1·16±0·17	ND	ND	ND
48	—	0·98±0·06	0·5±0·6	0·9±0·2	MNC≫PMN
48	Saline	1·09±0·06	0·5±0·0	0·8±0·3	MNC≫PMN
48	IL-1	1·45±0·11*	2·5±0·5	2·0±0·6	PMN≫MNC

Knee joints were injected with 5 μg Con A, a second injection of saline or IL-1 was given 42 h thereafter. PMN, Polymorphonuclear cell; MNC, mononuclear cell; ND, not done. Statistical analysis as described in Table 1.

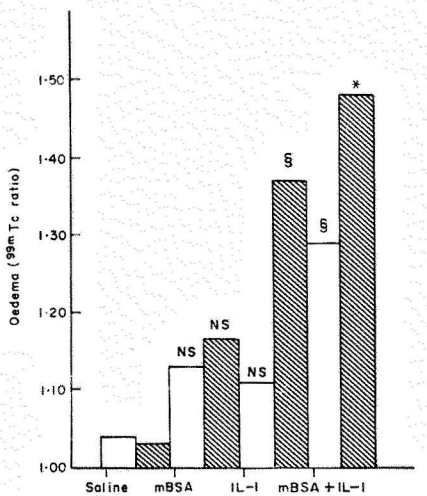

Fig. 4. Additive effect of IL-1 on antigen-induced exacerbation of the smouldering inflammation. Mice were intra-articularly injected with IL-1 (300 U), mBSA (10 ng) or a combination of both at week 2 of arthritis. Joint swelling was measured at 6 h and 24 h after induction of the flare-up. *, § Statistical analysis of ^{99m}Tc uptake as described in Table 1. ■, After 6 h; □, after 24 h.

not enhanced when antigen with IL-1 was used to elicit arthritis in a naive joint (data not shown).

DISCUSSION

The arthritic knee joint represents a hypersensitive area for systemic or local IL-1 of both types, IL-1α and IL-1β. IL-1 provoked an acute but transient relapse of the smouldering joint inflammation; severe joint swelling and marked neutrophil accumulation were evident. From our study, evidence emerged that a previously injured joint bearing a macrophage-rich synovial infiltrate displayed IL-1 hypersensitivity.

Unlike many other predisposed tissues, e.g. lung, vitreous tissue and rat ankles [17,27,28], the naive murine knee joint lacked oedema formation in response to IL-1. In general, full blown oedema is often found in the presence of a capillary permeability enhancer and a vasodilator, e.g. prostaglandin E_2 (PGE_2). In some tissues, addition of extra PGE_2 is necessary, while in other tissues sufficient PGE_2 is present anyway. Intradermal plasma leakage by IL-1 was only evident with added PGE_2 [29,30] and a similar situation probably exists for the murine knee joint. In contrast to a naive joint, an arthritic knee joint did display oedema formation upon IL-1 challenge. Surprisingly, even this oedema was not fully dependent on PGE_2, since indomethacin treatment only partly suppressed the oedema (Table 1). It is noteworthy that IL-1 is incapable of inducing PGE_2 synthesis in a naive joint [9,31], and it remains to be investigated whether this is different in an arthritic joint.

We and others have found that IL-1 induced neutrophil influx in a naive joint, although the mechanism is largely unknown. IL-1 itself is not chemotactic for polymorphonuclear cells (PMNs) *in vitro* [32,33], PMN influx is not dependent on prostaglandins [25], and leukotrien-B4 (LTB_4), a potent chemoattractant *in vitro*, is a poor chemoattractant in mice *in vivo* [34].

Neutrophil accumulation by IL-1 is more pronounced in an arthritic joint. One mechanism could be the enhanced expression of adhesion molecules (ICAM, ELAM-1) on vascular endothelium [35,36], or even vascular damage facilitating non-specific cell entrance. Although IL-1 sensitivity depends on the severity of the preceding inflammation (Fig. 3b), it is highly unlikely that the IL-1-induced flare-up is a result of the initial damage to the vessels by the primary inflammation, e.g. IL-1-induced flare-up exceeded the initial inflammation induced by Con A (Table 3). In addition, products of neutrophils, activated during the flare-up, might have accounted for the vascular damage causing indirect plasma leakage [37,38]. However, the IL-1-induced flare-up of arthritis in neutropenic animals denotes that neutrophil influx and oedema occurred independently of each other.

It seems likely that neutrophil influx is mediated by macrophage products, which are released upon IL-1 challenge. A recent report provided evidence that the IL-1-induced neutrophil accumulation in the peritoneal cavity in the rat is mediated by the resident macrophages [39].

Three novel macrophage-derived mediators are described with *in vivo* inflammatory properties, IL-8 (NAP-1) and the macrophage inflammatory proteins MIP 1 and MIP 2 [40–42]. IL-8 has recently been detected in synovial fluid and could be produced by the inflammatory exudate cells and synovium of arthritic patients [43,44]. IL-1-induced accumulation of neutrophils exceeded the monocyte accumulation [30,45] and this is in concordance with IL-8-induced chemotaxis [40]. Besides an acute neutrophil accumulation after injection, IL-8 also caused oedema formation, but the plasma leakage is prostaglandin— and neutrophil dependent [28,46]. IL-8 may therefore mediate the PMN influx into the arthritic joint after IL-1 injection, but is probably not solely responsible for the observed oedema formation.

The macrophage products, MIP 1 and MIP 2 injected into the footpads of mice resulted in a PMN-rich infiltrate [47,48]. It is not clear yet if MIP synthesis by the macrophages could be induced by IL-1 or that MIP could give rise to oedema formation.

Suggestive evidence is presented that IL-1 hyperreactivity in an arthritic joint is mediated by the macrophages in the synovial

infiltrate. IL-1 hypersensitivity occurred after 1 week, at the moment that macrophages become the predominant cells. Moreover, IL-1 hypersensitivity was demonstrated in the joint after Con A injection, which elicited mainly a mononuclear infiltrate. Depletion experiments eliminated both granulocytes (nitrogen-mustard treatment) and T cells (ALS treatment) as relevant cells in the IL-1 hypersensitivity. Finally, macrophages may generate IL-8 upon IL-1 challenge, which may mimic a number of the features of the IL-1 flare-up.

Exacerbations of a chronic T cell-mediated arthritis may occur in two ways: upon reactivation with the specific antigen and via IL-1. The first kind of flare-up depends on the specificity of the T cell infiltrate [20] and the balance between help and suppression. The IL-1 flare-ups are non-specific and may in fact occur at any moment that there is systemic generation of IL-1 by whatever process. It is tempting to speculate that at least part of the flare-ups in human RA are caused by this latter mechanism. Therapy should then be aimed at elimination or blockade of systemic IL-1. The recent cloning of the IL-1 receptor antagonist [49,50] may provide a useful tool for further research along this line. Another interesting IL-1 modulator for further study is TGF-β, which is known to downregulate or counteract many IL-1-driven processes, e.g. the IL-1-induced neutrophil adhesion to the endothelium [51].

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CHAPTER 9

ROLE OF INTERLEUKIN-1 (IL-1) IN ANTIGEN-INDUCED EXACERBATIONS OF MURINE ARTHRITIS

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Role of interleukin-1 (IL-1) in antigen-induced exacerbations of murine arthritis.

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Abstract

The mechanism underlying the chronic and intermittent course of rheumatoid arthritis is not elucidated. In the present study, the role of interleukin-1 (IL-1) was investigated in exacerbations of antigen-induced arthritis (AIA) in mice. A flare-up of the smouldering inflammation (week 3-4 of AIA) was inducible by injection of a small amount of methylated bovine serum albumin (mBSA), into the hypersensitive knee joint. Immunohistochemistry showed IL-1 expression in the synovial lining layer and in focal areas of the inflamed synovium during the flare-up. IL-1 was also measured in 1 hour culture supernatant of synovial tissue taken during the flare-up using a bioassay. The expression of both immunoreactive - and bioactive IL-1 in the hypersensitive joint, peaked around 6 hours after antigen (2 μg mBSA) injection and declined thereafter. Antigen rechallenge of the arthritic joint induced an acute joint swelling but not in the naive joint of the sensitized mouse although synovia of both joints produced IL-1 in response. Remarkably, a single intravenous injection of rabbit anti-IL-1 ($\alpha+\beta$) antibodies 1 hour before antigen rechallenge neutralized IL-1 in the joint. Anti-IL-1 treatment significantly reduced the antigen-induced joint swelling (30-40%) but did not affect the profound influx of polymorphonuclear (PMN) cells in the onset of the exacerbation. A profound relief of the inflammation (synovitis) was obtained by blocking IL-1 at day 4 of the exacerbation. Chondrocyte proteoglycan (PG) synthesis was markedly suppressed in the antigen challenged naive knee joints suggesting that this was a direct IL-1 effect as the inflammation was insignificant. Anti-IL-1 treatment maintained the PG synthesis in the antigen rechallenged joint which was highly suppressed in the control group. Furthermore, the enhanced PG breakdown in the antigen rechallenged joints was significantly decreased by anti-IL-1. We concluded that IL-1 is an important mediator produced during the onset of the exacerbations of arthritis causing cartilage destruction.

Introduction

There is increasing evidence to suggest that the pluripotent cytokine IL-1 plays an important role in the pathogenesis of inflammatory joint diseases, including rheumatoid arthritis (RA). IL-1 has been identified in the synovial membrane and cartilage-pannus junction of arthritic joints from RA

patients.[1,2] Moreover, numerous studies reported a striking corrrelation between the IL-1 levels in rheumatoid synovial fluid or plasma and the disease activity in these patients.[3-5] The two important features of arthritis namely inflammation and cartilage destruction, can be attributed to local IL-1 activity. In several mammalian species it was demonstrated that IL-1 injected into the synovial joints causes and arthritic insult.[6-8] *In vitro* studies showed that the chondrocyte PG synthesis was more susceptible for IL-1 than the chondrocyte mediated PG degradation.[9-11]

To prove the importance of IL-1 recent studies have adressed the effect of blocking IL-1 in experimental arthritis either by neutralizing IL-1 using antibodies or soluble IL-1 receptors, or by antagonizing the IL-1 binding to its receptor with IL-1ra and related proteins (M20). These different therapeutic approaches were able to diminish joint inflammation and also to reduced cartilage destruction in collagen-induced arthritis (CIA), immune complex arthritis (ICA), adjuvant arthritis (AA) and in the autoimmune prone MRL/lpr mice.[12-17] In murine AIA, anti-IL-1 treatment did not affect the acute joint inflammation (edema, influx of PMNs) nor the accelerated PG breakdown, yet the marked chondrocyte PG synthesis inhibition was completely prevented.[18-20] We were the first to demonstrate impressive reduction both in inflammation as well as in cartilage destruction in established CIA using rabbit anti-IL-1 polyclonal antibody treatment.[13] These studies at least suggest that IL-1 blockade is worth pursuing as a therapeutic strategy for human arthritis.

In 70% of the RA-patients the disease is characterized by an intermittent course of the inflammation.[21] In humans, a rise in plasma IL-1β levels followed the secondary flare-ups in RA and isolated peripheral monocytes spontaneously produced IL-1 during the flare-up.[3,22] In the present study, experiments were carried out to examine the role of IL-1 in the flare-up using an animal model. A flare-up of the smouldering inflammation in the chronic phase of AIA was inducible in a predictable and synchronized manner by injection of a small amount of mBSA into the hypersensitive knee joint.

Recently, Schwab et al.[23] demonstrated that IL-1 played a key role in the peptidoglycan-polysaccharide (PG-APS) polymer reactivation of *Streptoccal* cell wall (SCW)-induced arthritis. It cannot be excluded that at least part of the flare-reaction in this model was non-immunologically mediated.[24] Apart from PG-APS, flares can also be induced with cell wall derived lipopolysaccharide (LPS) and these fragments can directly activate macrophages. In AIA, T-lymphocytes as part of the residual inflammation in the arthritic joints, mediated the antigen-induced flare-up.[25,26] Antigen-stimulated T-cells could either produce IL-1 themselves or stimulate IL-1 production in other cells. In RA, it is still a matter of debate whether the arthritic process was driven by T-cells, immune complexes (IC), both, or by non-immunological pathways. Our study clearly demonstrate that IL-1 plays a key role in the antigen-induced exacerbation of murine arthritis.

Materials and methods

ANIMALS

Female New Zealand White rabbits, male Wistar rats, and male C57Bl/6 mice (Jackson Lab., USA) were fed a standard diet and tapwater freely.

CYTOKINES

Murine recombinant IL-1α and IL-1ß was a generous gift from I.G. Otterness (Pfizer Central Research, Groton CT, USA).

GENERATION OF RABBIT ANTI-MOUSE IL-1 ANTISERUM

Rabbits were immunization with murine recombinant IL-1α or IL-1β, according to the method as described by Hogquist et al.[27], with some modifications. In short, 250 μg IL-1 in 2.25 ml PBS was emulsified with 500 μl IMJECTTH ALUM (aluminium hydroxide, Pierce, Rockford, Illinois, USA). Each rabbit received 4 subcutaneous injections each 500 μl of the IL-1/ALUM suspension at the back of the animal and adjacent to each of these sites 4 500 μl CFA/PBS subcutaneously. Each rabbit recieved three booster injection of 50 μg IL-1/ALUM, adjacent injections with incomplete freunds adjuvant emulsified in PBS, and additionally 5 μg IL-1 intravenously, every 4-6 weeks. Ten days after every booster, 50 ml blood was aspirated, coagulated, decomplemented at 56°C, and stored at -70°C. The neutralizing capacity was 300 μg IL-1 per ml of the pooled antiserum.

PURIFICATION AND CHARACTERIZATION OF RABBIT ANTI-IL-1 ANTIBODIES

Immunoglobulins were purified by affinity chromatographic separation on a Protein G sepharose 4B (albumin binding region genetically deleted, Sigma, St Louis, Missouri, USA) column. Immunoglobulins were eluted with 0.1 M Glycine-HCl pH 3.0 and immediately neutralized with 50 mM Tris-HCl pH 8.0. Fractions were pooled, concentrated and dialysed against PBS at room temperature (RT). The anti-IL-1 antibodies showed no neutralizing reactivity against the other IL-1 subtype, IL-2, IL-4, IL-6, and TNF.

GENERATION OF RAT ANTI-MOUSE IL-1 ANTISERUM

Wistar rats were injected subcutaneously with 50 μg of murine recombinant IL-1β emusified in 0.5 ml CFA. After 2 weeks a booster injection was given of 25 μg IL-1 in 0.5 ml CFA. Rats were bled by orbital punction at a regular basis two weeks after the booster injection.

FLARE-UP OF ARTHRITIS

Mice of 8 to 12 weeks of age received 100 μg mBSA (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) emulsified in 100 μl complete freunds adjuvant (CFA) subcutaneously per animal, injections were divided over both flanks and footpads of the forelegs. Heat-killed ($2x10^9$) *Bordetella pertussis* organisms (National Institute of Public Health, Bilthoven, The

Netherlands) was administered intraperitoneally as an additional adjuvant. Mice received two booster injections of 50 μg mBSA/CFA in the neck region on day 7. Three weeks later, arthritis was induced by injection 6 μl of 60 μg mBSA in saline into the right knee joint cavity. At week 3 or 4 of arthritis, 0.2-2 μg mBSA was injected intra-articularly into the arthritic joints to induce a flare-up of the smouldering inflammation.

JOINT SWELLING

Mice were sedated by intraperitoneal injection of 4,5% chloral hydrate, 0.1 ml/10mg of body weight. Approximately 10 μCi ^{99m}Technetium pertechnetate (^{99m}Tc) in 0.2 ml saline was injected subcutaneously in the neck region. After 15 minutes the accumulation of the isotope in the knee was determined by external gamma counting and expressed as the ratio of the ^{99m}Tc uptake in inflamed over contralateral knee joint. A ratio higher than 1.1 indicates joint swelling.

IL-1 PRODUCTION BY SYNOVIAL TISSUE

Patellae were dissected with surrounding soft-tissue consisting out of the tendon, and synovium in a standardized manner. Each patella was incubated in 200 μl serum free RPMI 1640 (Dutch modification) medium with Glutamax-1 (Gibco BRL, Life Technologies, Schotland, UK) for 1 hour at room temperature. Dilutions of these wash-outs were tested for IL-1 and IL-2 bioactivity.

BIOASSAY FOR IL-1

IL-1 activity was measured in the one-stage bioassay for IL-1 as described by Gearing et al.[28]: The murine thymoma cell line EL-4 NOB-1 (ECACC, Porton Down, Salisbury, Wilts, UK) was used as an IL-1 specific cell producing IL-2 in response, in combination with the IL-2 sensitive CTLL2-cells (ECACC). The cells were plated out in concentrations of $1x10^5$/well NOB1-cells and $4x10^3$/well CTLL-cells in RPMI supplemented with 5% fetal calf serum (FCS), after 18-20 hour 0.5 μCi of ^{3}H-Thymidine (specific activity 20 Ci/mmol, Dupont, NENproducts, Boston, MA, USA) was added per well. Three hours later, cells were harvested and thymidine incorporation (NOB1 cell are thymidine kinase deficient) was determined. Detection limit of the assay was 0.1 pg/ml murine recombinant IL-1.[18]

ASSESSMENT OF PROTEOGLYCAN SYNTHESIS

Patellae (n=6), in a minimal amount of adjoining soft tissue (parts of synovium, tendon and muscle), were placed in 2 ml RPMI-1640 medium with gentamycin (50 μg/ml), and 40 μCi ^{35}S-sulfate. At the end of the 3 h incubation period, patellae were fixed in 10% formalin and subsequently decalcified in formic acid (4%), dissected and dissolved in 0.5 ml Lumasolve (Hicol, Oud-Beijerland, The Netherlands). The ^{35}S-content of each patella was measured by liquid scintillation counting and expressed as

counts per minute (CPM) or as a percentage of normal cartilage.

HISTOLOGY
Standard frontal sections (7 μm) of the whole knee joint, including both menici, crucial ligaments, and the patella, were prepared and mounted on gelatin (2%)-coated slides as previously described. Slides were stained with safranin-O and counterstained with fast green. Cartilage depletion (diminished red staining) and inflammation was scored by a blinded observer.

AUTORADIOGRAPHY
^{35}S-sulfate (100 μCi) (specific activity 1200-1400 Ci/mmol, NENproducts) was injected intraperitoneally 6 hours before dissection of the knee joints. After histological processing, 7 μm sections mounted on 0.5% gelatin coated slides were dipped in K5 emulsion (Ilford, Basildon, Essex, UK). The slides were developed and stained with hematoxylin and eosin after an exposure period of weeks. PG-synthesis was scored on the blackening of the chondrocytes by a blinded observer.

IL-1 IMMUNOSTAINING
Paraffin embedded sections were mounted on 2% gelatin coated slides, deparaffinized in xylene 2x5 minutes, rehydrated to deionized water. Endogenous peroxidase activity was blocked with 1% H_2O_2 in phosphate buffered saline (PBS) for 20 minutes at RT. Rinse in water. Slides were overnight incubation with the primary antibody, rat anti-IL-1β antiserum or irrelevant rat antibody, 1/150 diluted in PBS plus 5% non fat dry milk at 4°C in a humidified box. Wash in excess of PBS plus 0.1% tween-20, 3 x 10 minutes. Incubate slides with biotinylated secondary antibody, rabbit anti rat 1/200 diluted in PBS plus 5% non fat dry milk and 2% normal mouse serum for 1 hour at RT. Wash again in PBS/Tween, 3x10 minutes. Prepare avidin biotin/ peroxidase conjugated complex according to Vectastain's directions for preparation (Vectastain ABC kit, Vector Lab Inc, USA) and incubate slides with the ABC reagent diluted in PBS plus 5% non fat dry milk for 30 minutes at RT. Wash again in PBS/Tween. Develop slides with 0.5 mg/ml diaminibenzidine (DAB) in Tris-HCl pH 7.6 and 0.02% H_2O_2 for 10 minutes. Counterstain with haema-toxyline for 15 seconds. Anti-IL-1 antiserum preincubated on IL-1β coated plates, 8 x 20 minutes at RT, or normal rat serum was used as a negative control. For control of IL-1ß specificity, antiserum was preincubated on IL-1α or BSA coated plates.

TREATMENT OF MICE WITH ANTI-IL-1 ANTIBODIES
Mice received 200 μl of standard dose of 1 mg purified rabbit anti-IL-1α antibody and 1 mg of anti-IL-1ß antibody intra-venously into the *orbita plexus*, 1 hour before induction of the flare-up. These antibodies had an excellent half-life of more than 3 days. Total neutralizing capacity of the

received dose was about 32 ng of both subtypes of IL-1 as was tested in the IL-1 bioassay (NOB-1), and blocked the effect of 1 ng of IL-1α or ß on proteoglycan synthesis completely and that of 10 ng IL-1 partially *in vivo* (not shown). Normal rabbit IgG's or polyclonal anti-ovalbumin antibodies, served as controls for non-specific effects of the treatment.

Results

IL-1 expression in antigen-rechallenged knee joints of chronic arthritis.

At the time of antigen-rechallenge, week 3 of AIA, avidin-biotin immunohistochemistry using rat antiserum to IL-1ß failed to demonstrate IL-1 on whole knee joint sections (not shown). A single intra-articular injection of a small amount of antigen (2 μg mBSA) caused a transient expression of cell associated IL-1ß. The number of IL-1ß positive cells was small in the joint sections taken at 1 hour, high at 3 hour, low but still evident at 6 and 12 hours, and absent at 24 hour after the antigen-rechallenge (not shown). The IL-1ß signal was predominantly localized in the synovial lining layer and frequently in focal areas in the inflamed synovium (Figure 1). No immunostaining was evident using normal rat serum, nor using anti-IL-1ß antiserum preincubated on microtiter plates coated with murine recombinant IL-1ß (Figure 1). In some sections, chondocytes and the exudate polymorphonuclear cells were non-specifically stained.

A single intra-articular injection of mBSA into hypersensitive joints (week 3 AIA) caused a dose-related joint swelling at 6 hour (Figure 2). Synovial tissue culture supernatants demonstrated increased levels of IL-1 bioactivity 6 hours after antigen rechallenge in the tested dose range of 0.06 to 2 μg mBSA (Figure 2). Time course experiments demonstrated a rapid onset of joint swelling increasing at least upto 24 hours after antigen (2 μg mBSA) rechallenge of AIA (Figure 3). The level of bioactive IL-1 in synovial tissue culture supernatants of arthritic joints was high at 3 and 6 hour after antigen rechallenge (Figure 3). The expression of IL-1 was more transient compared to the joint swelling. At 24 hour after antigen injection, IL-1 released by the synovial tissue was low yet joints were profoundly swollen. Joint swelling was not inducible with 2 μg mBSA injected into naive joints of sensitized animals (Figure 3). Interestingly, the antigen challenged naive joints released IL-1 in levels and time course comparable to the rechallenged arthritic joints (Figure 3).

Role of IL-1 in the antigen-induced exacerbation of the chronic joint inflammation.

At the time of antigen rechallenge, week 3 of AIA, swelling of the arthritic joints subsided, the inflammatory exudate cells disappeared but joints had a mild ongoing synovitis (Table 1). Intra-articular injection of 2 μg mBSA into the arthritic joints caused an acute transient joint swelling. At day 2 of the flare-up a Tc-ratio of 1.22 $\pm$ 0.14 was measured.

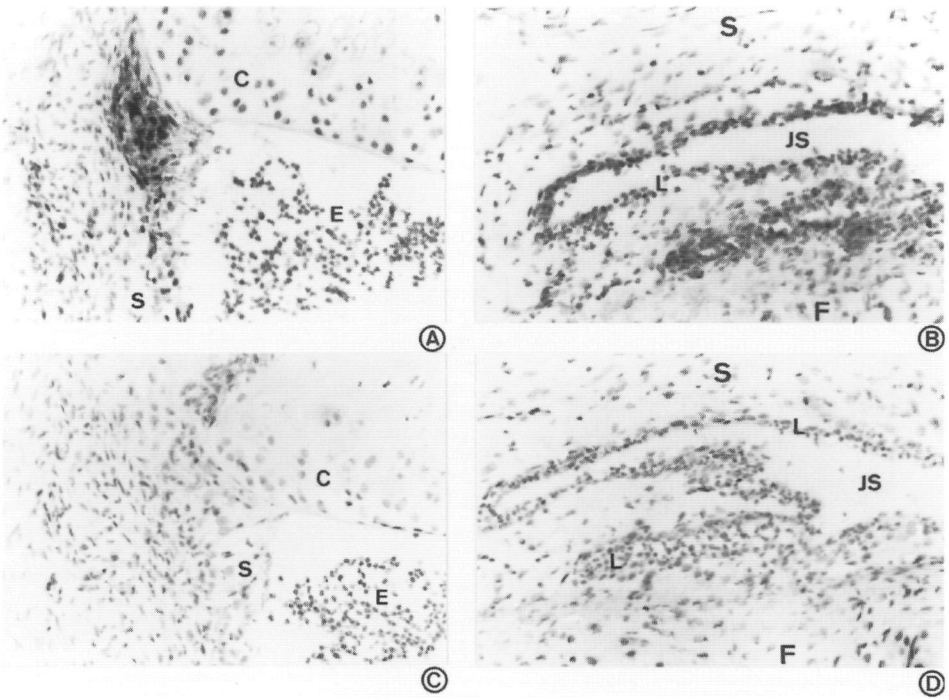

Figure 1: Immunoperoxidase localization of whole knee joint sections showing cells stained for IL-1β (magnification x 250) in focal areas of the inflamed synovium (a) and in the synovial lining layer (b). Antigen (2 μg mBSA) was injected intraarticularly at week 3 of AIA and joints were dissected 3 hours thereafter. Serial sections stained with normal rat serum (c) or with anti-IL-1β antiserum preincubated on IL-1β coated microtiter plates (d) served as negative controls. Abbreviations: C, cartilage; E, exudate; JS, joint space; L, lining layer; S, synovium.

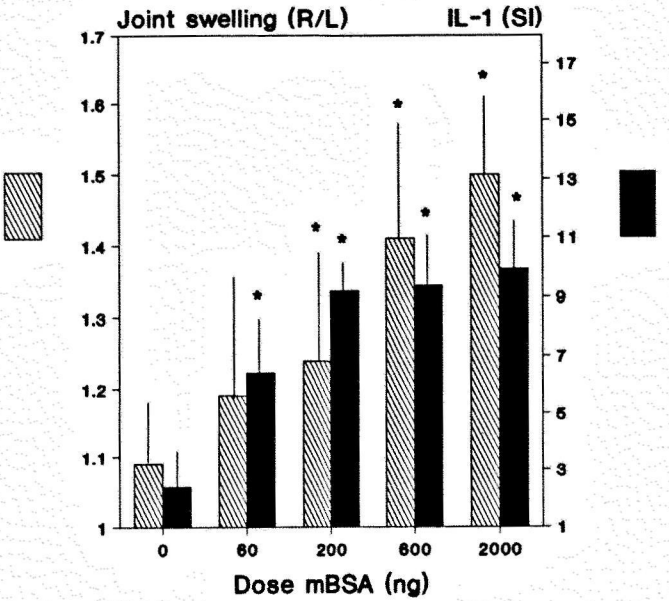

Figure 2: Dose related joint swelling and IL-1 production in antigen rechallenged hypersensitive knee joints. Antigen (0.06-2 μg mBSA) was injected intra-articularly into arthritic joints at week 3 of AIA. Joint swelling was measured 6 hour after the antigen rechallenge by external gamma counting of ^{99m}Technetium pertechnetate uptake. Values represent the mean ratio of arthritic (R) over the naive contralateral joint (L) (± S.D.) of 7 animals. A 1/80 fold dilution of culture supernatants of synovial tissue taken 6 hours after antigen rechallenge was tested for IL-1 using a cell proliferation assay. Values represent the mean stimulation index (± S.D.) of 6 individual synovia. Highest signal (SI) is comparable with the response to 4 pg/ml of IL-1 in the assay (10.000 CPM). Statistical significance was tested by using the Wilcoxon's rank sum tests. $P<0.05$ (*) was considered significant.

The same antigen dose injected into the naive contralateral joints of these arthritic animals did not cause joint swelling (Table 1).

To investigate the role of *de novo* synthesized IL-1, neutralizing polyclonal anti-IL-1 ($\alpha+\beta$) antibodies were injected intravenously one hour before antigen rechallenge. Blocking IL-1 decreased joint swelling at 6 hour (-29%) and significantly reduced joint swelling at 24 hour (-41%) after antigen rechallenge as compared to the untreated mice (Table 1). This was a consistent finding in all experiments, mean joint swelling of 1.60 ± 0.21 in the untreated mice (n=47), 1.52 ± 0.24 in the normal rabbit IgG treated mice (n=68), and 1.37 ± 0.29 in the anti-IL-1 treated mice (n=75) at day 1 after antigen rechallenge. Antigen injection also caused a marked influx of polymorphonuclear neutrophils (PMN) into the cavity, and exacerbated the ongoing synovitis in the hypersensitive joint (Table 1). A moderate inflammation was inducible in the naive contralateral joints of these sensitized animals with the same antigen dose (2 μg mBSA) used (Table 1).

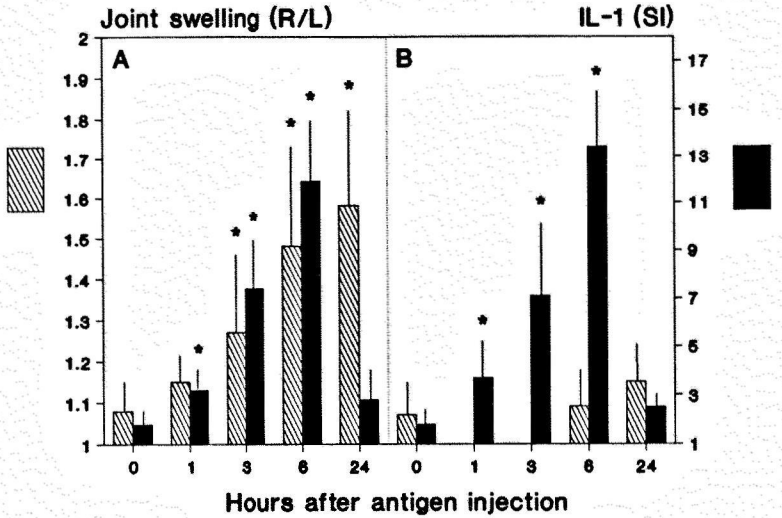

*Figure 3: Time related changes in joint swelling and IL-1 production in antigen rechallenged hypersensitive knee joints (A) and in naive joints of sensitized mice (B). Antigen (2 μg mBSA) was injected intra-articularly and both joint swelling and IL-1 production by the synovial tissues was measured (as described in figure 2) at several timepoints thereafter in 6 animals each. Statistical significance was tested by using the Wilcoxon's rank sum tests, *=P<0.05.*

Table 1: Role of IL-1 in the antigen-induced exacerbation of the smouldering joint inflammation.

Antigen	Pre-treatment	Joint swelling Tc-ratio (R/L)		Histology Exudate		Histology Infiltrate		
		6 h (n=8)	24 h (n=12)	6 h (n=7)	24 h (n=8)	6 h (n=7)	24 h (n=7)	96 h (n=8)
-	None	1.07 (.07)	1.05 (.08)	0.0 (0.0)	0.0 (0.0)	0.8 (0.6)	0.4 (0.4)	0.4 (0.2)
+	None	1.49 (.26)	1.70 (.10)	1.4 (0.5)	1.7 (0.9)	1.7 (0.8)	1.6 (0.8)	1.4 (0.9)
+	NRS	1.46 (.15)	1.66 (.20)	1.8 (0.9)	1.8 (0.8)	2.1 (1.1)	1.9 (0.6)	1.4 (0.9)
+	AIL1	1.35 (.23)	1.41 (.17)*	1.6 (1.0)	1.8 (0.8)	2.1 (1.2)	1.6 (0.8)	0.5 (0.1)*
+J	None	ND	1.06 (.08)	0.3 (0.3)	0.6 (0.4)	0.2 (0.3)	0.4 (0.1)	ND

Arthritic knee joints rechallenged with 2 μg mBSA at week 3 of AIA (+).
Antigen injected into the naive contralateral knee joints.
Number of mice per group are indicated between brackets.
Mice pretreated with normal rabbit antibodies (NRS) or anti-IL-1(α+β) antibodies (AIL1).
Joint swelling was measured at six and 24 hours after the antigen injection by ^{99m}Technetium (Tc) uptake (S.D.).
Histological analysis of cell exudate (PMNs) in the joint cavity at 6 and 24 hour after antigen injection. Arbitrary scoring from 0 (no cells) to 3 (large number of inflammatory cells) by a blinded observer.
Histological analysis of cell infiltrate (at 6 and 24 hour) and synovial thickness (at 96 hour) after antigen injection. Statistical significance was tested by using the Wilcoxon's rank sum tests. P<0.05 (*) was considered significant.

Anti-IL-1 ($\alpha+\beta$) antibody pretreatment had no effect on the number of inflammatory cells (PMN) in the 6 and 24 hour exudate and on the extent of the synovitis at these time points (Table 1). A marked amelioration of the synovitis was observed at day 4 after antigen-rechallenge in the anti-IL-1 treated group (Table 1, Figure 5). This was a consistent finding in 3 out of a total of 4 experiments. Synovitis (1.55 $\pm$ 0.69) in the normal rabbit IgG treated mice (n=21) was significantly higher than in the anti-IL-1 treated mice (0.63 $\pm$ 0.23, a reduction of 59%).

Role of IL-1 in the flare-up related cartilage pathology.

Chondrocyte PG synthesis, markedly suppressed the first weeks of AIA, returned to a normal rate at week 3 of AIA. A marked suppression of the PG synthesis was evident at day 1 (-28%) and day 2 (-23%) after antigen rechallenge in patellar cartilage as was measured by ^{35}S-sulfate incorporation *ex vivo* (Table 2). Pretreatment of mice with anti-IL-1 antibodies completely prevented the proteoglycan synthesis suppression to occur (Table 2). Autoradiography of whole knee joint sections also demonstrated reduced ^{35}S-sulfate incorporation by the chondrocytes in cartilage of the arthritic joints from day 1 upto day 4 after antigen rechallenge (Table 3, Figure 4). Blocking the IL-1 activity with antibodies significantly enhanced the PG synthesis in the antigen rechallenged mice (Table 3, Figure 4).

Table 2: Effect of anti-IL-1 treatment on PG synthesis suppression and PG degradation in antigen challenged joints.

		Proteoglycan metabolism (CPM $\pm$ SD)		
Antigen[1]	*Treatment*[2]	*Synthesis*[3] *Day 1* *(n=7)*[4]	*Synthesis*[3] *Day 2* *(n=7)*	*Degradation*[5] *Day 1* *(n=6)*
-	None	1118 $\pm$ 104	1238 $\pm$ 289	360 $\pm$ 99
+	None	739 $\pm$ 54 (-28%)	958 $\pm$ 251 (-23%)	ND
+	NRS	732 $\pm$ 103 (-35%)	913 $\pm$ 343 (-26%)	148 $\pm$ 39 (-59%)
+	AIL-1	1068 $\pm$ 143* (-4%)	1519 $\pm$ 778 (+23%)	226 $\pm$ 40*(-37%)
-	None[1]	1153 $\pm$ 258	ND	ND
+	None[1]	860 $\pm$ 170*(-25%)	ND	ND

[1] Arthritic knee joints rechallenged with 2 μg mBSA at week 3 of AIA (+).
[1] Antigen injected into the naive contralateral knee joints.
[4] Number of mice per group are indicated between brackets.
[2] Normal rabbit antibodies (NRS) or anti-IL-1 ($\alpha+\beta$) antibodies (AIL-1) injected 1 hour before antigen rechallenge.
[3] ^{35}S-sulfate incorporation into patellar cartilage ex vivo. Values between brackets indicate the inhibition of PG synthesis as a percentage of the unchallenged joint. Representative of two experiments.
[5] ^{35}S-sulfate content in patellar cartilage. ^{35}S-sulfate was injected subcutaneously 24 hour before antigen injection.
Statistical significance was tested by using the student's t test, *=P<0.05.

Table 3: Histological analysis of the anti-IL-1 treatment effect on PG synthesis and cartilage PG depletion in antigen challenged joints.

Antigen[	Treatment[	Proteoglycan synthesis[			Depletion ⊢
		Day 1 (n=7) ⊢	Day 2 (n=8)	Day 4 (n=6)	Day 4 (n=8)
-	None	2.0 ± 0.9	2.2 ± 0.3	2.1 ± 0.9	0.4 ± 0.8
+	None	0.5 ± 0.3 (-77%)	ND	ND	2.1 ± 0.6
+	NRS	0.7 ± 0.4 (-70%)	0.9 ± 0.6 (-60%)	0.9 ± 0.7 (- 60%)	1.9 ± 0.9
+	AIL-1	1.4 ± 0.7*(-30%)	1.9 ± 0.7*(-14%)	2.5 ± 0.5*(+17%)	0.3 ± 0.5*
-	None]	1.3 ± 0.6	ND	ND	0.0 ± 0.0
+	None]	0.5 ± 0.3*(-63%)	ND	ND	0.4 ± 0.2

[Arthritic knee joints rechallenged with 2 μg mBSA at week 4 of AIA (+).
] Antigen injected into the naive contralateral knee joints.
⊢ Number of mice per group are indicated between brackets.
⫣ Normal rabbit antibodies (NRS) or anti-IL-1 (α+β) antibodies (AIL-1) injected 1 hour before antigen rechallenge.
⫞ Autoradiography of whole knee joint sections. ^{35}S-sulfate incorporation into patellar cartilage in vivo as scored arbitrary from 0 (no black spots) to 3 (strong blackening as seen in the growth plates). Values between brackets indicate PG synthesis inhibition.
⊨ Histological analysis of patellar cartilage PG depletion as scored arbitrary from 0 (strong safranin-O staining = no depletion) to 3 (unstained cartilage = strong depletion) by a blinded observer.
Statistical significance was tested by using the Wilcoxon's rank sum tests, *=P<0.05.

Interestingly, injection of 2 μg mBSA into the naive contralateral joints of these mice also resulted in a significant suppression of the PG synthesis at day 1 (Table 2 and 3).

Enhanced proteoglycan degradation (-59%) was found in the hypersensitive joints 1 day after antigen rechallenge as was measured on ^{35}S-sulfate prelabeled patellar cartilage *in vivo* (Table 2). The accelerated degradation could be significantly reduced by anti-IL-1 treatment but was still remarkable (-37%) (Table 2).

Histology taken at week 4, but not at week 3, of AIA showed full recovery of the cartilage matrices (Figure 5). Antigen (2 μg mBSA) rechallenge caused severe PG depletion of the cartilage matrices as was shown on safranin-O stained whole knee joint sections (Table 3, Figure 5). Pretreatment with anti-IL-1 antibodies, but not with control IgG, significantly ameliorated the cartilage pathology at day 4 of the antigen-induced exacerbation (Table 3, Figure 5). Anti-IL-1 (α+β) pretreatment completely prevented the antigen-induced cartilage depletion in the hypersensitive joints in 4 out of 5 experiments. Antigen injection into the naive contralateral joints of the arthritic mice caused only minor cartilage depletion (Table 3).

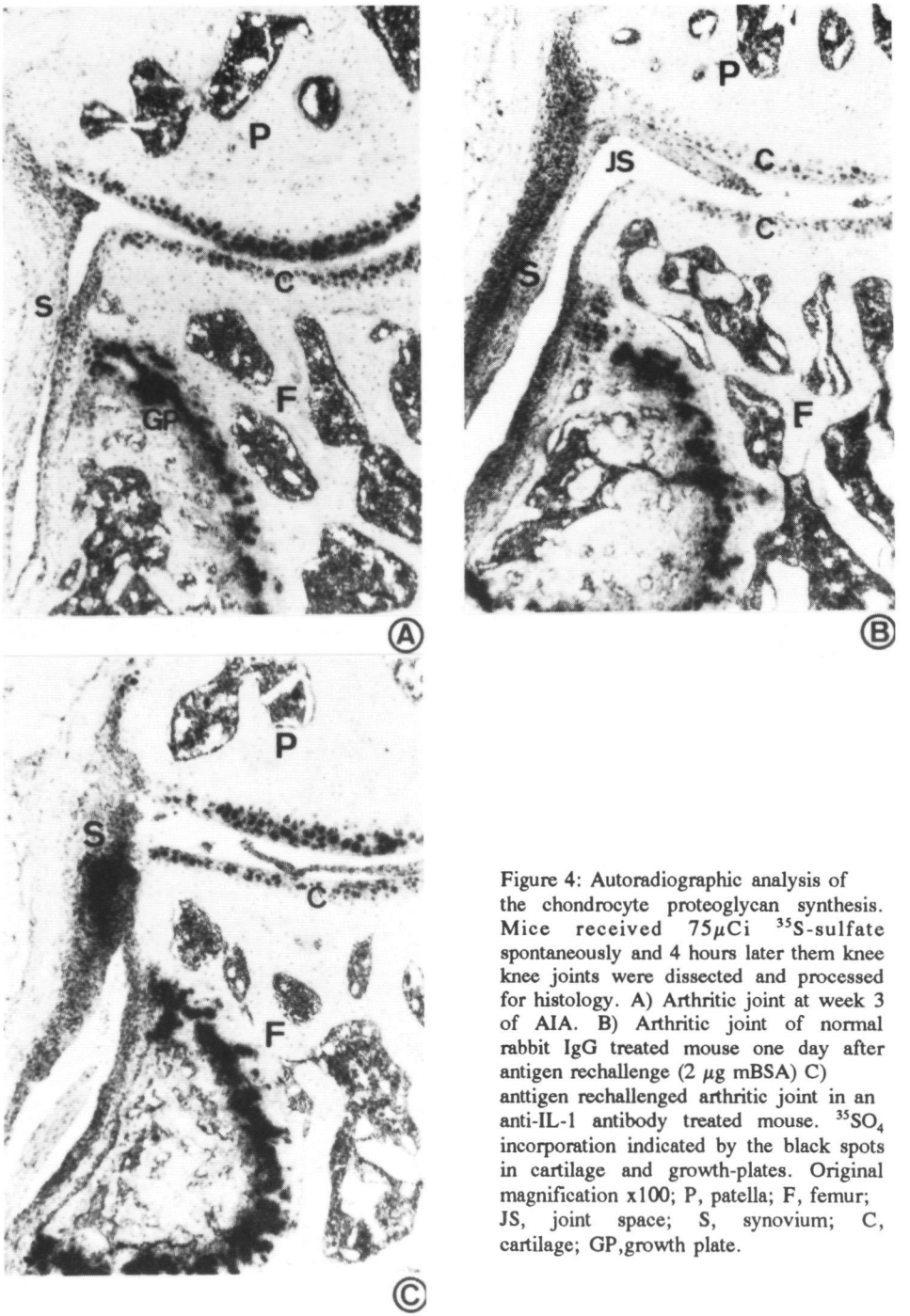

Figure 4: Autoradiographic analysis of the chondrocyte proteoglycan synthesis. Mice received 75μCi ^{35}S-sulfate spontaneously and 4 hours later them knee knee joints were dissected and processed for histology. A) Arthritic joint at week 3 of AIA. B) Arthritic joint of normal rabbit IgG treated mouse one day after antigen rechallenge (2 μg mBSA) C) anttigen rechallenged arthritic joint in an anti-IL-1 antibody treated mouse. $^{35}SO_4$ incorporation indicated by the black spots in cartilage and growth-plates. Original magnification x100; P, patella; F, femur; JS, joint space; S, synovium; C, cartilage; GP,growth plate.

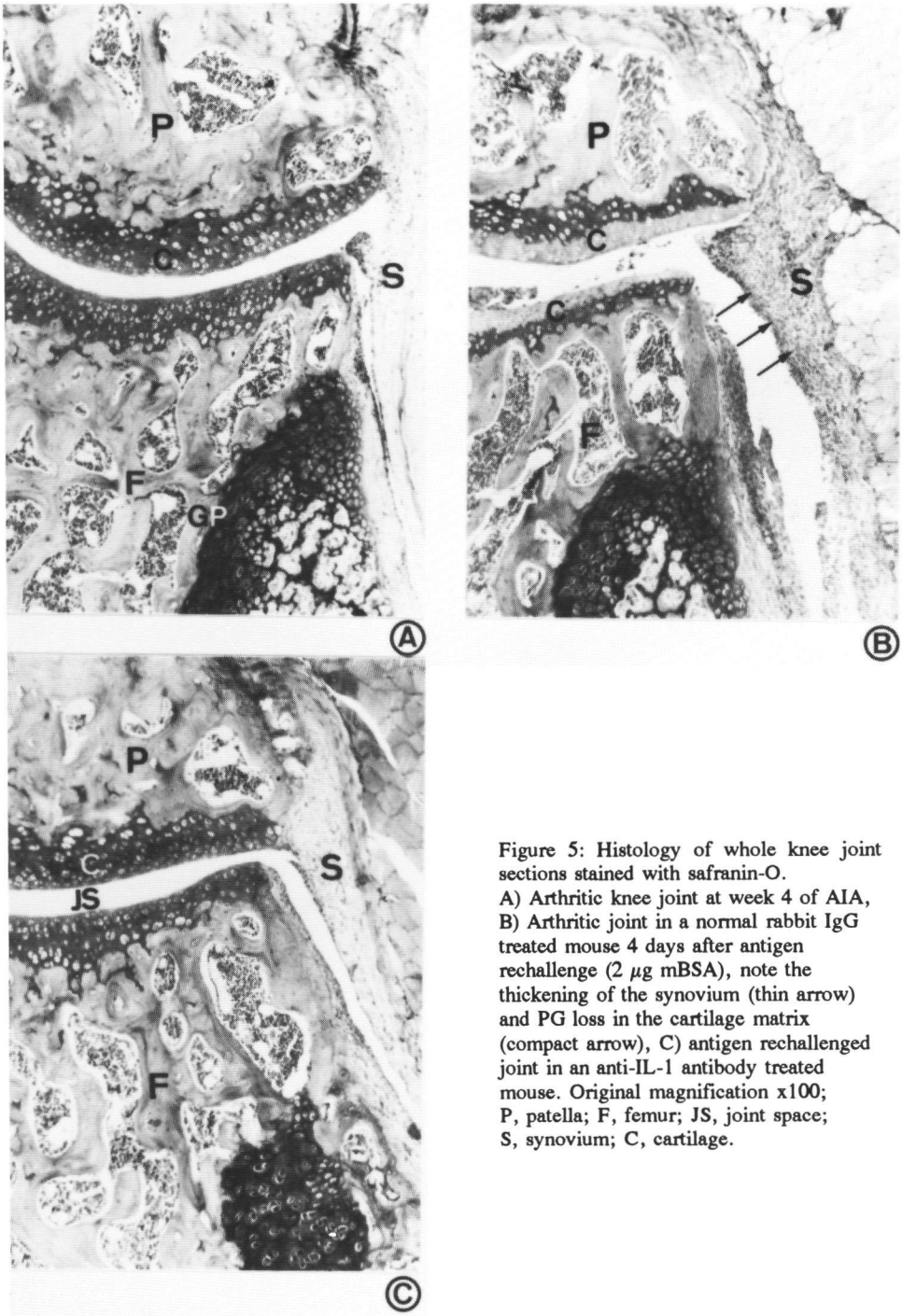

Figure 5: Histology of whole knee joint sections stained with safranin-O.
A) Arthritic knee joint at week 4 of AIA, B) Arthritic joint in a normal rabbit IgG treated mouse 4 days after antigen rechallenge (2 μg mBSA), note the thickening of the synovium (thin arrow) and PG loss in the cartilage matrix (compact arrow), C) antigen rechallenged joint in an anti-IL-1 antibody treated mouse. Original magnification x100; P, patella; F, femur; JS, joint space; S, synovium; C, cartilage.

Discussion

This study clearly demonstrates that IL-1 is an important determinator in antigen-induced exacerbations of murine arthritis. In the conducted experiments, pretreatment of mice with rabbit polyclonal antibodies directed against murine recombinant IL-1α and IL-1β markedly alleviated joint swelling and ameliorated cartilage pathology of the antigen rechallenged joints.

Using avidin biotin immunohistochemistry we showed IL-1β expression in the synovium of antigen rechallenged joints, predominantly located in the lining layer, in distinct cell clusters, and positive cells were more sparsely distributed in the deeper area. An identical localization was found in synovial membranes of rheumatoid arthritis for IL-1α and IL-1β.[1,2] The expression of IL-1ra was identical to IL-1 in the rheumatoid synovium although the number of positive cells was considerably smaller.[1] We found an identical kinetic for both the expression of bioactive and immunoreactive IL-1 in hypersensitive joints after antigen rechallenge. This indicates that IL-1 inhibitors (e.g. IL-1ra), although they may be produced, were not responsible for the transient IL-1 expression in the flare-up.

IL-1 was inducible in both virgin and hypersensitive joints of sensitized mice but not in joints of normal mice.[18] This shows that the stimulation of IL-1 was not a property of the antigen but that either lymphocytes or immune-complexes were involved. IL-1 production in rheumatoid synovial membranes was considerably higher in membranes possessing lymphocyte aggregates compared to membranes with a disperse infiltrate.[29] These focal areas consisted out of B cells, plasma cells and $CD4^+$ T cells in the center and macrophages at the periphery. T helper type 2 cells but also B cells as almost all accessory cells (e.g. macrophages) can produce IL-1. We did not quantitatively compare the IL-1 production in antigen challenged naive and hypersensitive joints, but only in the later we found distinct staining of cell clusters.

The antigen dose used to elicited a flare-up of AIA was not arthritogenic in normal knee joints of sensitized mice. This suggest that the anigen-induced IL-1 production was too low to induce joint inflammation. We previously demonstrated that a single injection of 10 ng IL-1 could not induce joint swelling and cell exudate in naive joints but induced a short lasting flare-up of the inflammation in hypersensitive joints of AIA as was displayed by increased joint swelling and a marked cell exudate.[8,30] A highly likely explanation for the hypersensitive state could be the increased expression of adhesion molecules (VCAm-1, ICAMs) in the inflamed synovium.[31] In the present study, we found using anti-IL-1($\alpha+\beta$) antibodies that the cell exudate and the joint swelling for the greater part (60-70%), were not IL-1 mediated in the antigen-induced exacerbation of AIA, and this suggest that other mediators are involved in this process.

Indisputable evidence was presented that CD4 positive T-cells are mediating the antigen-induced exacerbation of murine arthritis.[32] It was

concluded from the full blockade of the flare-up by anti-Ia (histocompatibility type II antigens) antibodies that antigen presentation was of importance.[26] Although IL-1 is capable of activating lymphocytes, we found that the IL-1 induced exacerbation of AIA was not lymphocyte mediated.[30] Evidence is emerging that IL-1 does not play a prominent role in the antigen induced T-cell activation.[33,34] For instance, Schwab et al.[35] demonstrated that reactivation of SCW arthritis by the T-cell superantigen, toxin shock syndrome toxin-1, was neither IL-1 nor TNFα dependent and this contrasted with the peptidoglycan polysaccharide reactivation suggesting that the latter was not solely T-cell mediated.

We selectively blocked the TH1 derived IL-2 and the TH2 derived IL-4 with antibodies and this markedly reduced joint swelling, cell exudate and infiltrate during the antigen-induced flare-up.[32] Co-injection of large amounts of IL-2 or IL-4 with the antigen significantly enhanced cell exudate and it was concluded both cytokines acted proinflammatory in the hypersensitive joint.

A more pivotal role of IL-1 was found in immune-complex (IC) mediated inflammations.[36,37] Recently we developed an immune-complex mediated arthritis model (ICA) by passive immunization in mouse.[14] In this model, IL-1 and complement activation acted synergistically in the onset of the inflammation. Striking anti-inflammatory effects using anti-IL-1 antibodies or IL-1 receptor antagonist protein (IL-1ra) were also found in CIA, even when treatment was started in already established disease.[13] In contrast, anti-IL-1 treatment had no effect on the onset of AIA.[18-20] CIA is probably more IC- than T-cell mediated.[38,39] For instance, although CIA could be transferred by T-cells and IC, a combination resulted in a more pronounced inflammation.[40] However, AIA and the subsequent hypersensitive state of the joints could be transferred with antigen (mBSA)-specific T-cells to naive recipients.[41] Up till now, we were unable to transfer the disease (AIA) by B-cells or immunoglobulins (unpublished data). The importance of IL-1 could be related to the type of inflammation or the site of the inflammation.[16,42]

Antigen rechallenge of the hypersensitive joints induced a marked inhibition of the chondrocyte proteoglycan synthesis for at least 4 days. This was not a property of the antigen as PG synthesis suppression was not inducible in joints of non-immune mice.[18] The flare-up of the inflammation was not responsible for the suppression of the chondrocyte synthetic function for two reasons. First, antigen injection into naive joints of sensitized mice induced both IL-1 production and inhibition of the PG synthesis without a significant joint inflammation. Second, blocking IL-1 with neutralizing anti-IL-1 ($\alpha+\beta$) antibodies protected the chondrocyte synthetic function without affecting the onset of the flare-up. We previously demonstrated a key role of IL-1 in the inhibition of proteoglycan synthesis in several murine arthritis models i.e. CIA, AIA, and ICA.[13,18,19,43]

The enhanced cartilage degradation in the antigen-challenged arthritic joints was for about 50% IL-1 mediated. In ICA and CIA, the anti-IL-1 also significantly reduced proteoglycan degradation but this was probably related to the alleviation of the joint inflammation.[13,44] A more likely explanation for the protective effect of anti-IL-1 treatment on the flare-up is a higher vulnerability for IL-1 induced degradation of cartilage in the recovery phase of arthritis (Manuscript in preparation). Pelletier et al.[45] presented evidence for an enhanced number of IL-1 receptors on chondrocytes in human OA cartilage and showed that the IL-1 required for half-maximal metalloproteinase stimulation was 3-4 times lower compared to normal cartilage.

Protection of the chondrocyte synthetic function and reduction of the enhanced PG degradation probably resulted in the observed amelioration of the cartilage pathology at day 4 after antigen rechallenge. Mini-osmotic pumps releasing 30 μg IL-1ra per hour (Synergen) into the peritoneal cavity from day 2 before antigen rechallenge upto 7 days thereafter, verified the anti-IL-1 antibody effects (not shown).

Another impressive effect of the anti-IL-1 treatment was the reduction in the synovial thickness (hyperplasia) at day 4 of the exacerbation. IL-1 is a potent growth factor for synovial fibroblasts and this can explain this anti-IL-1 effect.[46] Synoviocytes can actively participate in the local hypersensitivity for a number of reasons. First, synovial fibroblasts (synoviocytes) are accessory cels by their capacity of antigen presentation and IL-1 expression.[47,48] Second, adhesion molecules can be induced by cytokines on type B synoviocytes and this may facilitate leukocyte recruitment.[49,50] Moreover, unrestricted synoviocyte proliferation caused arthritis in animals and synovial tissue implants caused cartilage destruction.[51,52] For this, the reduction of the synovial hyperplasia in the antigen rechallenged joints could be responsible for the amelioration of arthritis. Interestingly, IL-4 is an major regulatory molecule in the inflamed tissue by inducing IL-1ra and MCP-1 and reducing PGE_2 release and synoviocyte proliferation.[53,54]

This study indicate that IL-1 is involved as key mediator in the pathological changes of cartilage not only in IC-mediated inflammation but also in T-cell mediated inflammations. Our study strengthen the need for further research of the role of IL-1 even in the case IL-1 is not involved in the onset of the inflammation.

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CHAPTER 10

FINAL CONSIDERATIONS

10.1 Outline of the general discussion.

Rheumatoid arthritis is a systemic illness with polyarticular manifestations of chronic inflammation. The levels of IL-1 in the circulation correlates with the index of disease activity (chapter 1.3). In animal models of arthritis, IL-1 expression is associated with onset and course of the joint inflammation (chapter 1.4). IL-1 production can take place in the arthritic joint and at the systemic level, for example, the IL-1 production by peritoneal macrophages and bone marrow cells in rat adjuvant arthritis and the bone marrow cells in collagen-induced arthritis (CIA). The spontaneous IL-1 production by human peripheral blood monocytes coincided with exacerbations of the inflammation in RA patients [1]. It remains to be seen whether the IL-1 production at the local or at the systemic level modulates the course of the joint inflammation. For this, the effect of intra-articular or systemic administration of IL-1 on the inflammation was studied in experimental arthritis (chapter 10.2).

The role of endogenous IL-1 in the arthritic process will be evaluated in chapters 10-3 and 10-4. It is known that IL-1 takes part in both immuno-regulatory and inflammatory processes, however, the involvement of both processes and their interplay may differ between the arthritis models. Furthermore, anti-IL-1 was either given in a prophylactic regimen, before or during the immunization, or in a therapeutic regimen, started after full blown immunity, in the different studies. This makes it difficult to define at which level (process) of the disease anti-IL-1 interferes with.

In RA, the spontaneous IL-1 production by isolated synoviocytes was maintained by *de novo* TNF production and for this anti-TNF treatment was expected to mimic anti-IL-1 treatment. The role of TNF in the arthritic process in the animal models is reviewed (chapter 10.5) and the future perspective of anti-IL-1 treatment of RA is evaluated and compared with recent clinical findings of the anti-TNFα treatment (chapter 10.6).

10.2 Effect of exogenous IL-1 on experimental arthritis.

ACCELERATION OF (COLLAGEN-INDUCED) ARTHRITIS

Daily subcutaneous injections of human recombinant IL-1ß during the immunization against heterologous type II collagen, accelerated onset, increased incidence, and enhanced the progression of CIA in mice and rats [2-6]. The circulating antibody levels against type II

collagen (chicken or bovine) are comparable between sensitized non-arthritic mice and age-matched, IL-1 injected arthritic animals. Furthermore, IL-1 was only able to promote arthritis in susceptible mouse strains and not in genetically resistent strains although the later had comparable antibody levels against type II collagen [6]. The circulating antibody levels against heterologous type II collagen did, however, not correlate with the number of affected joints or the histological grade [7]. CIA is more likely to be initiated by cross-reactive antibodies against autologous type II collagen [8]. For this, it is possible that IL-1 promotes CIA in prone animals by an enhanced anti-collagen antibody response against autologous collagen but this was not assessed in these studies.

T-cells are also involved in CIA, since anti-IL-2 receptor antibody treatment effectively suppressed murine CIA [9]. Systemically administered IL-1 did not induce arthritis in 'susceptible', $L3T4^+$ T cells depleted mice [6], however, IL-1 injections in adoptively (T + B cells) transferred mice increased the anti-type II collagen antibody levels and caused a transient arthritis [3,6]. A possible explanation for this is that IL-1 changes the humoral response through T-cell activation. Another explanation could be that IL-1 stimulates T-cells to cause arthritis. As circumstantial evidence, prolonged treatment with human TNFα started before the immunization increased the T-cell response (DTH-reaction) against type II collagen rather than the antibody response and simultaneously aggravated CIA in rats [10]. On the other hand, IL-1 or TNF may lower a threshold in animals with nascent illness. Exogenous IL-1 may bypass the IL-1 inhibitory effects of IL-4 and IL-10, both Th2 subset derived cytokines [11-16], and by this initiates inflammation.

Crucial in this model is the immune response against autologous type II collagen. In normal cartilage the collagen fibrils are surrounded by proteoglycan aggregates and this makes it unlikely that type II collagen epitopes are exposed to B- or T-cells. The epitopes becomes available during cartilage breakdown. A likely mechanism since in IL-1 challenged prone animals (established immunity (day 14)) arthritis developed within the first day after IL-1 injection [6]. Interestingly, IL-1 is more potent on cartilage of the ankle joint than the knee [17] and since the ankle joint is the prevalent site of CIA, this implies that IL-1 exerts its potentiating effect on CIA by direct action on cartilage rather than on the immunity. A likely explanation also for the IL-1 acceleration of the spontaneous arthritis in autoimmune MRL/lpr mice [18].

EXACERBATION OF THE JOINT INFLAMMATION

In *Streptococcal* cell wall (SCW)-induced arthritis in rat, intra-articular IL-1 injections into the arthritic ankle joint exacerbated the smouldering inflammation. Clear-cut aggravation of arthritis was found after repeated IL-1-induced flares [19]. We showed that local or systemic administration of IL-1 induced a flare-up of the smouldering inflammation in antigen-induced arthritis (AIA) in mice (AIA) (chapter 8). The IL-1 induced flare-up of AIA was more transient compared to the IL-1 induced flare-up of SCW-arthritis but in both models marked cartilage degradation was shown (preliminary data) and this was not induced by IL-1 in naive knee joints (chapter 3). In humans, affected cartilage of osteoarthritic - and RA-patients is more vulnerable to IL-1 induced proteoglycan loss than normal cartilage [20].

The antigen-induced exacerbation of SCW-arthritis with peptidoglycan polysaccharide (PG-APS) and AIA with methylated bovine serum albumin (mBSA) are both T-cell dependent. In AIA, the antigen-induced flare-up could be blocked by pretreatment with either anti-lymphocyte sera and anti-Ia antibodies and this was recently confirmed with anti-CD4 treatment. Anti-lymphocyte pretreatment did not prevent the IL-1 induced flare-up of chronic AIA [21]. This and the IL-1 induced flare-up of zymosan-induced arthritis (ZIA) and concanavalin A-induced inflammation in knee joints, implies that not T-cells but more likely the macrophage rich infiltrate is responsible for the observed IL-1 hypersensitivity (chapter 8).

Studies in the rabbit failed to note joint swelling in spite of the heavy infiltration of leukocytes [22]. We found no swelling in the murine knee joint up to 10 ng IL-1α/knee (measured as Tc) (chapter 3). Stimpson et al. [19], measured an ankle joint swelling to murine IL-1α in the rat and found a measurable response at around 5 ng of IL-1. Chandrasekhar et al. [23] compared the ankle with the knee and found a good swelling response from 1-10 ng huIL-1β i.a. into the ankle whereas 5-10 μg were required for a similar swelling response in the knee. This indicate that the ankle is more sensitive to IL-1 than the knee joint. IL-1 also induce a profound edema in highly vascularized tissues such as lung and vitreous tissue, suggesting that the IL-1 induced plasma leakage in previously inflamed joints is related to impaired endothelial vessel wall integrity or neovascularization.

10.3 Effect of anti-IL-1 treatment on joint inflammation in experimental arthritis.

NON-T-CELL MEDIATED ARTHRITIS MODELS.

Van Lent et al. [24] developed an immune complex arthritis model (ICA) induced in the mouse by passive immunization with rabbit anti-lysozyme antibodies followed by i.a. injection of low amounts of poly-LL-lysine coupled lysozyme (3 μg). Pre-treatment with rabbit anti-IL-1 (α + β) polyclonal antibodies markedly suppressed joint swelling and influx of polymorphonuclear leucocytes (PMN) but did not prevent thickening of the synovial membrane [24]. Joint inflammation could not be induced with aBSA into passively immunized (rabbit anti-aBSA antibodies) mice. The difference is that poly-LL-lysine coupled lysozyme is a potent IL-1 inducer in the joint whereas aBSA is not. In ICA, both IL-1 and complement activation acted synergically in the induction phase of arthritis. Depleting macrophage-like synovial lining layer cells by dichloromethylene diphosphonate encapsulated liposomes reduced IL-1 production and subsequently prevented arthritis [25,26]. In RA, the numbers of synovial tissue macrophages significantly correlates with the degree of joint erosions [27].

Zymosan is also a potent trigger of IL-1 production in monocytes whereas the IL-1 receptor antaginist protein (IL-1ra) could not be induced by zymosan in the same cells [28-30]. Intra-articular injected zymosan caused an acute joint inflammation and cartilage destruction. In zymosan-induced arthritis (ZIA), the anti-IL-1 treatment significantly reduced joint swelling (maximal -24%) but the influx of PMN was not affected (chapter 7). A conflicting result with the potent anti-inflammatory effect of anti-IL-1 on zymosan-induced pleurisy and subcutaneous inflammation (tissue chamber) [30,31]. It is concluded that although zymosan (alternatively) activates complement factors and stimulates IL-1 production, zymosan induced joint inflammation by an IL-1 independent pathway.

T-CELL INVOLVED ARTHRITIS MODELS

In the above mentioned arthritis models (ICA,ZIA) no active immunization against the trigger is required and there is no evidence that T-cells are involved in the onset of the disease.

Wooley et al. [32] treated CIA with human IL-1ra by daily intraperitoneal injections (3-300 μg) started at day 14 of the

immunization, and this delayed onset and reduced the incidence of CIA in DBA/1 LacJ mice. The IL-1ra treatment significantly reduced the levels of circulating anti-collagen type II antibodies (-30%) but this was unrelated to the anti-inflammatory effect of IL-1ra in CIA. Van den Berg et al. [33] showed that a single intraperitoneal injection of neutralizing rabbit anti-IL-1 (α + ß) polyclonal antibodies completely protected mice in developing arthritis and almost completely attenuated arthritis when injected in the progressive phase of the disease [33]. Anti-IL-1 decreased the number of affected joints, diminished synovitis, and markedly reduction cartilage pathology [33]. Intraperitoneal injections of monoclonal antibody against IL-1ß three times per week, started at day 3 of the immunization, prohibited mice to develop arthritis [34]. Treatment started at day 21 of the immunization significantly reduced the incidence of arthritis (80% --> 30% at day 60) and joint destruction (X-ray assessment). Van den Berg et al. [33] showed that a full recovery of established arthritis is only possible after blocking both IL-1 subtypes.

Again the humoral and cellular responses to homologous type II collagen were not assessed in these studies. The immunity against homologous collagen does not have to develop synchronic with the immune response against heterologous type II collagen.

The same IL-1ra treatment regimen as used in CIA, either started during or after the immunization, did not modulate AIA nor the humoral and cellular immune-responses against the antigen in rat [32]. Injections of huIL-1ra, 4 mg/Kg subcutaneously every six hours, had no effect on the acute joint inflammation at day 3 of ovalbumin-induced arthritis (AIA) in the rabbit [35]. Faherty et al. [36] showed ongoing antigen specific immune responses *in vivo* after treatment with IL-1ra or with the monoclonal anti-IL-1RI antibodies (35F5). IL-1ra and 35F5 antibodies are blocking the IL-1 type 1 receptors on, for example, Th2-cells. Th1-cells predominantly expressed the type II IL-1 receptor which has a low affinity for IL-1ra and this suggests that the Th1 cells could supply sufficient help for the development of the humoral response [37,38]. The antigen specific T-cells in patients with reactive arthritis are predominantly of the Th1 cell-type [39].

A single injection of anti-IL-1 (α + ß) polyclonal antibodies intravenously one day before arthritis induction completely neutralized *de novo* synthesized IL-1 during the acute phase of murine AIA, however, did not reduce the acute joint swelling (day 1 and 2) nor the exudate of neutrophils (chapter 5,6). The acute inflammation remains IL-1 independent even when the antigen dose was lowered in AIA. A significant reduction of the joint

swelling and synovitis was evident at day 4 and 7 of AIA by the anti-IL-1 pre-treatment. Repeated IL-1ra injections (10mg/kg every 3-6 hours) or even continuous delivery of IL-1ra (37.5 μg/hour) by implantation mini-osmotic pumps intra-peritoneally did also not prevent the joint inflammation in murine AIA (chapter 7).

The anti-inflammatory effect of IL-1ra might depend on the localisation of arthritis, the ankle in CIA and the knee joint in AIA. IL-1ra is more efficacious in prevention of IL-1 induced suppression of the proteoglycan synthesis in cartilage of the ankle joint than in cartilage of the knee joint [17].[1] The IL-1 receptor type I expression of chondrocytes is considerably lower in the ankle compared to the knee joint. Zymosan injected into naive knee joints induced an acute joint inflammation restricted to that joint in normal mice but induced joint inflammation in the ankle of the draining paw in type II collagen sensitized mice. Anti-IL-1 antibody treatment prevented the zymosan accelerated CIA [77]. IL-1 derived from the zymosan-injected knee joint probably increased the type II collagen epitopes expression and this triggers the inflammatory reaction in the ankle.

The anti-inflammatory effect of the anti-IL-1 in other arthritis models implies that IL-1 is involved in the immune development in these animals. The disorder in MRL/lpr autoimmune mice is characterized by excessive lymphocyte proliferation and development of autoantibodies most prevalently directed against dsDNA and collagen type II. Either treatment of nascent or established disease with a soluble IL-1 receptor type I (sIL-1RI), with equal affinities for IL-1α and β, reduced autoantibodies production (-75%), alleviated inflammation, ameliorated cartilage destruction and arrested the development of polyarthritis [40]. Treatment with M20, an IL-1ra related protein, inhibited the joint swelling, peri-articular erythema, limping of the rats, and reduced the mononuclear cell infiltrate in joints of adjuvant arthritis (AA) in the rat [41]. In a standardized AA model, treatment with sIL-1RI prevented the disease symptoms, even when starting therapy in the established disease. sIL-1RI inhibited the disorder from spreading to the non-injected extremity, probably by its strong immunosuppressive effects on the production of autoreactive anti-type II collagen antibodies (-43%). Furthermore, this treatment suppressed joint swelling in the secundary lesions [40].

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1 Plenary lecture, KE. Kuettner, IAIS, Vienna, 1993.

T-CELL DEPENDENT ARTHRITIS MODELS.

The arthritis models may differ in their relative contribution of both the humoral and T-cell immunity in the disease.

In the chronic phase of SCW-arthritis in rats, an i.v. injection of the superantigen (TSST-1) resulted in an acute reactivation of the inflammation. IL-1ra treatment failed to reduce the acute joint swelling [42]. In the chronic phase of murine AIA, a T-cell dependent flare-up of the smouldering inflammation could be induced with low amounts of antigen (2 μg mBSA), and anti-IL-1 (α + ß) antibody treatment significantly reduced joint swelling (-30%) without affecting neutrophil infiltration. Blocking IL-1 significantly reduced the flare-up related synovitis (chapter 8).
In rabbbit AIA, IL-1ra treatment started after arthritis induction moderately reduced the synovium collagen content (-22.4%) [43]. These studies suggest that T-cell mediated inflammation are for the major part, if not at all, IL-1 independently regulated.

MISCELLANEOUS INFLAMMATION MODELS

The inflammation in peptidoglycan polyssaccharide (PG-APS) reactivation of SCW-arthritis was less acute and lasted several days compared with the antigen-induced flare-up of murine AIA. Cyclosporin pretreatment of the animals prevented the TSST-1 flare-up completely but the suppression was less complete in the first two weeks of the PG-APS induced flare-up, indicating that PG-APS induced flares are not strictly T-cell dependent [42]. In the PG-APS reactivation model, Schwab et al. [44] found an ambiguous role for endogenous IL-1 in the onset of the flare-up. Blocking IL-1 with IL-1ra for the first 6 hours of the flare enhances joint swelling but blocking for 24 hours suppressed the joint swelling for 55% at the most. This indicates that IL-1 is pro-inflammatory, but at the same time may initiate anti-inflammatory homeostatic responses. For example, an intravenous injection of IL-1 induced a flare-up and induced feedback mechanisms able to suppress a subsequent PG-APS reactivation [44].

Strain differences were also noted in the synthesis of IL-1ra by the same stimuli. Macrophages of rats susceptible for PG-APS induced SCW-arthritis, produced equivalent amounts of Il-1 but reduced amounts of IL-1ra in comparison to resistant rat strains [45]. Collagen-induced arthritis is also strain specific and DBA/lac mice may also have an impaired (feedback) suppressor mechanism

probably at the levels of macrophage IL-1/IL-1ra expression as exogenous IL-1 could initiate arthritis. T-cell derived lymphokines as IL-4 and IL-10 of subset TH2 are known to antagonize IL-1 at more than one level [11-16]. Moreover, evidence emerged that IC stimulate monocyte to synthesize both IL-1 and IL-1ra [24,28,46,47].

10.4 IL-1 induced cartilage pathology and its role in arthritis.

IL-1 INDUCED SUPPRESSION OF PROTEOGLYCAN (PG) SYNTHESIS

We (chapter 2 and 3) showed that murine recombinant IL-1 markedly suppressed PG synthesis in murine articular cartilage *in vitro* and *in vivo*. Chandrasekhar et al. [48] found similar results with huIL-1ß in rat. Arner et al. [49] measured proteoglycan synthesis in the rabbit and found that 100 ng of huIL-1ß/knee is required for significant suppression. The IL-1-induced proteoglycan synthesis suppression is probably mediated by nitric-oxide (NO) production in the chondrocytes.[2] The IL-1 induced inflammation is too mild to be involved in the PG synthesis suppression (chapter 3).

Topographical variations in chondrocyte responses towards *in vivo* IL-1 challenge are described [50]. The inhibition of proteoglycan synthesis at the periphery of patellar cartilage was considerably smaller than that at the central part of the patella, femur and tibial plateau. After repeated IL-1 injections, prolonged suppression of chondrocyte proteoglycan synthesis in both the central part of the patella and the medial side of the femur was demonstrated by autoradiography [50]. It was hypothesized that environmental influences (e.g. distance to the synovium) and loading conditions predisposed certain areas in the joint. As circumstantial evidence, short-term immobilization of the knee joint in extension after IL-1 injection prevented Il-1 induced proteoglycan synthesis inhibition [51]. Dingle et al. [52] showed that the femur was more easily depleted than the tibia. Page-Thomas et al. [53] reported topographical differences in the proteoglycan synthetic recovery rate which is slower in patellae than in tibial cartilage of rabbits.

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ROLE OF IL-1 IN THE SUPPRESSION OF PG SYNTHESIS IN EXPERIMENTAL ARTHRITIS

We showed that blocking IL-1 activity with either rabbit polyclonal antibodies or with human IL-1ra prevented the suppression of chondrocyte PG synthesis in both murine AIA and ZIA (chapter 5-7). This protective effect was not due to a relieve of the inflammation since anti-IL-1 had no anti-inflammatory effect in these models. Starting the anti-IL-1 treatment 6 hours after arthritis induction, so at the time of profound neutrophils influx, still completely prevented the PG synthesis suppression. Normalization of PG-synthesis was also shown in arthritis models in which anti-IL-1 treatment had a strong anti-inflammatory effect such as murine CIA and ICA. ICA induction in neutropenic mice inhibited the PG synthesis even in the absence of exudate and infiltrate. This parallels with expression of IL-1 bioactivity in joints during ICA elicited in neutropenic mice.

IL-1 INDUCED CARTILAGE PROTEOGLYCAN DEGRADATION

Intra-articular injections of IL-1 caused a short lasting but marked loss of articular cartilage proteoglycans in rabbits, rats, and mouse [48,48,52,54-58](chapter 3) and the insult was more profound after repeated injections (chapter 4). Human IL-1ß injected into a normal, non-inflamed rabbit knee joints caused a short-lasting (48 hours) leucocyte infiltrate into the synovium and exudate into the joint space and resulted in significant cartilage proteoglycan loss [59]. Henderson et al. [60] showed that intravenous injection of IL-1ra inhibited both proteoglycan loss and inflammation that follows after intraarticular huIL-1ß injection in rabbits. The same extent of joint inflammation induced by zymosan or LPS as seen after IL-1 did not induce proteoglycan loss (chapter 3)[59]. Furthermore, IL-1 injected into neutropenic rabbits still caused cartilage proteoglycan depletion [62,62]. These observations suggest that IL-1 induced inflammation (neutrophil influx) and IL-1 induced cartilage loss are unrelated. Intra-articularly injected huIL-1α or β caused infiltration of approximately equal numbers of PMNs and monocytes [63]. Human TNF preferentially stimulated the infiltration of monocytes [64,65] but this did not cause cartilage proteoglycan depletion.

The relief of joint inflammation and amelioration of PG degradation after anti-IL-1 treatment coincided in murine CIA and ICA. For this reason, a direct link between IL-1 and the observed arthritic cartilage changes could not be proven in these models.

IL-1ra treatment did not diminishe cartilage PG loss in rabbit AIA [43,66]. Anti-IL-1 antibodies also did not reverse the accelerated PG degradation in the first two days of murine AIA (chapter 7). The normalization of PG synthesis after anti-IL-1 treatment probably accounts for the observed amelioration of the cartilage loss in murine AIA (chapter 5 and 7). We cannot exclude that the reduction of synovitis by anti-IL-1 in the acute phase of AIA and the antigen-induced exacerbation of AIA causes a reduction of the PG breakdown at later time points in the disease.

In ICA, pretreatment with anti-IL-1 antibodies suppressed the accelerated PG degradation by 30-50%. ICA in neutropenic mice failed to show PG degradation, since IL-1 was still generated in the joint, it proves that the PG degradation was not a direct consequence of IL-1 in this model [67].

The role of the neutrophils is intriguing. The cartilage proteoglycan loss induced by the reversed passive arthus reaction in the rabbit joint did not occur in neutropenic animals [68]. In contrast, cartilage loss induced by AIA was unaffected in neutropenic rabbits and mice [62,69]. Although the PMNs are highly destructive for cartilage *in vitro* through the release of elastase, evidence for a role of elastase *in vivo* is lacking [70,71].

10.5 Role of TNF in arthritis models

Recently, transgenic mouse lines have been generated which express the human TNF gene [72]. These transgenic mice show dysregulated patterns of TNF expression and develop a chronic polyarthyritis, which is demonstrated by a swelling of the ankles, impaired movement, hyperplasia of the synovial membrane, and PMN and lymphocyte inflammatory infiltrates by week 3 of age. In addition, pannus formation, articular cartilage destruction and massive production of fibrous tissue were observed in the advanced stages of the disease.

A transgenic animal with over-expression of IL-1 has not yet been reported. Moreover, IL-1 involvement in the TNF

transgenic mouse is not evaluated although Brennan et al. [72] clearly demonstrated a direct link between IL-1 production and TNF activity in the rheumatoid synovium.

The role of TNF in murine CIA has been extensively studied. Blocking TNF *in situ* has been attempted by pretreatment with a number of agents; anti-TNF (α + β) antibodies, soluble human p55 TNF receptor, and a recombinant human soluble TNF receptor FC fusion (rhuTNF:Fc) protein [72-77]. In general, blocking TNF in the imunization period resulted in a marked (50-60%) reduction of paw swelling, significant reduction of the number of severely affected joints (erosions, synovitis), and reduced the incidence significantly from 86% to 28%. IL-Ira treatment also reduced incidence from 80% to 10% and ameliorated arthritis to the same extent (see previous chapters). Both anti-TNF and anti-IL-1 treatments are reported to delay onset of arthritis [76,77]. In contrast to the anti-IL-1 treatment, anti-TNF treatment only moderately (-15%) improved the clinical score (edema, erythema, joint rigidity).

Van den Berg et al. compared both the anti-IL-1 and anti-TNF treatments in collagen arthritis. Anti-TNF treatment resulted in an arrest of the disease whereas anti-IL-1 treatment resulted in complete recovery even in fully established arthritis [78]. These results with anti-TNF antibodies are identical to the results obtained by Wooley et al. using the rhuTNF:Fc fusion protein, reduced arthritic score by a combination of less severe disease and fewer involved joints [77]. Multiple anti-TNF antibody injections at the onset of arthritis (day 1) also arrested joint swelling, reduced the clinical score and the number of severely affected joints [74]. No therapeutic effect was found when anti-TNF treatment was started in more established arthritis (month 2 after immunization) although anti-IL-1 antibody treatment suppressed arthritis completely [33,34,75]. In CIA, blocking IL-1 seemed to be more effective than blocking TNF, and this could not be explained by reduced anti-type II collagen antibody levels as huTNF:Fc fusion protein was as effective as IL-1ra in this respect [32,77].
TNF and IL-1 are locally proinflammatory and intra-articular injections caused a transient neutrophil influx which lasted several hours in the joint [79]. Cooper et al. [80] demonstrated that intra-articular injections of human TNFα in either knee or ankle, accelerated onset of CIA, increased incidence and joint swelling in Wistar rats. In passively immunized rats with antibodies against type II collagen, i.a. injections of TNFα into the ankle resulted in an acute transient joint swelling [80]. Brahn et al. [10]

demonstrated that a human TNFα administered by osmotic pumps from the start of the immunization, aggravated both the clinical and radiographic parameters of CIA in rats. In mice, prolonged treatment with murine TNFα by daily i.p. injections from the day of immunization, resulted in an increased incidence and severity of CIA in mice [76].

Lewthwaite et al.[3] treated ovalbumin-sensitized rabbits with rat anti-rabbit TNFα monoclonal antibodies during the onset of AIA and joint swelling (-39%) and leucocyte infiltration (-42%) were reduced but the histopathology of the synovial lining was not affected. Moreover, anti-TNF treatment did not diminish articular cartilage depletion although the inflammation was reduced by anti-TNF treatment. We treated murine AIA with anti-TNF antibodies and we found no effect on the joint swelling or exudate (chapter 7). Moreover, anti-TNF treatment did not protect the proteoglycan synthesis in AIA (chapter 7) and CIA [81]. In the chronic phase of murine AIA, i.a. injections of muTNFα caused a short-lasting flare of the smouldering inflammation and cartilage destruction (not shown).

In SCW-arthritis, TNF seemed to play a more prominent role, Schwab et al. [42,44] demonstrated that the the flare-up induced with PG-APS but not with the superantigen TSST-1 could be suppressed by either IL-1ra or anti-TNF antibody treatment.

Joint inflammation was IL-1 and to a lesser extent TNF dependent in CIA and the PG-APS flare of SCW-induced arthritis. In more T-cell mediated arthritis (AIA, TSST-1 flare of SCW) both IL-1 and TNF seemed to play a minor role. A clear discrepancy was found in murine ICA: IL-1 played a major role in inflammation and pathology whereas anti-TNF antibody treatment had no effect on these parameters of arthritis (unpublished data). A possible explanation for this could be the IL-1 inducing property of the cationic antigen used in this model.

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[3] Lewthwaite JC, Blake SM, Vetterlein O, Foulkes R, Bodmer M, Hardingham TE, Henderson B: The effect of anti-TNFα monoclonal antibodies on the induction of antigen-induced arthritis in the rabbit. ORS abstract, Feb 21-24, 1994.

10.6 Perspective of anti-IL-1 treatment of rheumatoid arthritis.

The applicability of inhibition of arthritis by anti-IL-1 and anti-TNF reagents to human arthritis is currently an important area of research.

Recently in the first clinical trial, the chimeric monoclonal anti-TNF antibody (cA2) was highly effective in the treatment of RA. A cumulative dose of 20 mg/Kg of cA2 had no adverse effect and resulted in a clearcut improvement of clinical and laboratory parameters in 19/19 patients [82,83]. Although the anti-TNF treatment of RA showed good efficacy in this open trial, the proper controls were lacking. For instance, control chimeric antibodies or proteins were not included in the trial. Furthermore, no prove was presented that the treatments reduced cytokine bioactivity, to show that the cytokines were indeed blocked. A nice example was found in RA-patients treated with IL-6 neutralizing monoclonal antibodies. Marked clinical improvements were obtained but unexpectedly these antibodies neutralized IL-6 *in vitro* but *in vivo* functioned as a carrier and elevated IL-6 levels were found in these patients [84].

The rational of targetting TNF in rheumatoid arthritis is presented in the previous chapter: TNF overexpression in mice causes arthritis [72]. TNF plays a role in the onset of inflammation in CIA. IL-1 production by rheumatoid synoviocytes could be blocked by anti-TNF-antibodies [73]. However, false conclusions may be drawn using bioassays to measure IL-1 synthesis. For instance, no bioactive IL-1 was present in the exudate of the *Bordetella pertussis* vaccine induced inflammation in sensitized mouse. However, the IL-1 activity seemed to be antagonized in the bioassay as measurements of the immunoreactive IL-1 showed increased synthesis during the inflammation [29]. Brennan et al. [73] did not measure immunoreactive IL-1 and the effect of anti-TNF antibodies on the spontaneous IL-1 production by synoviocytes could be due to increased synthesis of e.g. IL-1ra by aspecific stimulation of the cells [47]. An important point to adress is that in the experimental arthritis models clear evidence for an incontestable TNF --> IL-1 cascade is lacking.

Nevertheless, the results obtained in the first clinical trial with the chimeric monoclonal anti-TNF antibodies are impressive. Clearly the experimental arthritis models showed that blocking IL-1 is as effective as blocking TNF and in many models even better. Thompson, R.C. presented preliminary results of phase I trials with

IL-1ra in RA-patients.[4] IL-1ra treatment in 15 RA-patients reduced the number of tender joints. No adverse effects were noted, it did not affect the immune responses or evoked autoantibodies against IL-1ra in these patients [85,86]. The use of IL-1ra has major drawbacks because molecular excess of 1000 was needed to fully block IL-1. The novel technic of gene-transfer was employed and local expression of huIL-1ra in the synovial joint was able to block the effects of i.a. IL-1 injections [87-89]. Gene-transfer could be a suitable new strategy for RA, expression is restricted to the inflamed area and this will narrow down possible side effects. Using recombinant chimeric proteins of high avidity anti-IL-1 antibodies with human FC may increase the efficacy of the anti-IL-1 antibody treatment of RA.

It is highly likely that RA forms a heterogenous group with complex profiles and interrelationships of cytokines. Mulligan and Ward [90] showed an organ specificity with respect to the requirement for TNF and IL-1 in IgG immune complex-induced injury. In the lung, both TNF and IL-1 are important, whereas in similar induced injury in the skin IL-1, but not TNF is required. A similar observation was made with zymosan-induced inflammation. In the joint, inflammation was IL-1 independent and this is in conflict with the more prominent of IL-1 in the zymosan-induced pleurisy and in the subcutaneous tissue chamber model [30,31]. The influx of neutrophils was almost completely attenuated. In previous chapters we mentioned frequently another feature that of the cytokine sensitivity between the different joints, the ankle joints more sensitive for IL-1 induced injury than the knee joints, but also gender differences in the IL-1 response of cartilage are reported. The femoral head cartilage of the female rat to be more sensitive to IL-1 induced proteoglycan synthesis suppression, but male cartilage more vulnerable to IL-1 induced degradation [91]. The net IL-1 effect on proteoglycan content was the same in both sexes. The inhibition of proteoglycan synthesis and acceleration of proteoglycan degradation by IL-1 are mediated by independent post receptor pathways [92,93].

Can the RA-patient group be divided into either TNF or IL-1 producers. This was substantiated by the following findings: First, in rheumatoid arthritis, levels of IL-1 and TNF in synovial fluids do not correlate (chapter 1.3 of this thesis). Second, IL-1 and TNF

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[4] Thrird international workshop on cytokines, Stresa, Italy, 1991

production of rheumatoid synovium do not correlate [73,94]. Third, IL-4 exposure of some rheumatoid synovia resulted in complete inhibition of the TNF production while IL-1 was only slightly suppressed [95]. Fourth, evidence is presented that both syntheses are independently regulated, e.g. the TNF but not the IL-1 production was completely attenuated in LPS tolerized animals [96,97].

RA-patients could also be divided with regard to their anti-inflammatory response on non-steroidal antiiflammatory drugs (NSAIDS). A link was suggested with the anti-inflammatory effects after NSAIDS or anti-IL-1/TNF treatment in certain experimental arthritis models (CIA and AA). Moderate or no anti-inflammatory effects were found with anti-IL-1/TNF treatments in arthritis models which are less responsive to NSAIDS, e.g. AIA in rabbit and mice. Suggesting that IL-1-induced prostaglandin (PG)E_2 production may be more important in the former models of arthritis and that anti-IL-1 blocked the synthesis of prostaglandin or the related eicosanoids. This is supported by the observation that NSAIDs inhibit the acceleration of CIA by exogenous IL-1 but that indomethacin did not suppress the IL-1 induced flare of AIA [4,21].

One should also consider the impact of NSAID-treatment on the cytokine profile in RA-patients. Prostaglandins are potent feed-back mediators of the IL-1 and TNF synthesis. For example, fysiological levels of prostaglandins decreased TNF synthesis for about 50% in rheumatoid synovium [98]. Indomethacin treatment of AIA resulted in marked suppression of joint swelling and synovial PGE_2 levels but increased the lymphocyte numbers infiltration and aggravated cartilage proteoglycan loss [99]. During the course of RA, patients may shift into higher IL-1 or TNF producers due to the effect of NSAIDS.

In summary, the clinical trials with anti-TNF and anti-IL-1 lacked radiographic analysis of cartilage pathology. Targetting IL-1 in RA may have a larger impact as we clearly demonstrated that IL-1 was more potent in inducing cartilage pathology than TNF. Comparative studies in three different experimental arthritis models (AIA, CIA, ICA) were performed, all demonstrating a more important role for IL-1 than TNF. All the evidence points to IL-1 as a pivotal mediator in the inhibition of chondrocyte proteoglycan synthesis during arthritis. A marked reduction of the accelerated proteoglycan degradation was shown in animal models with IL-1 playing a key role in the inflammation.

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CHAPTER 11

NEDERLANDSE SAMENVATTING

Rheumatoide arthritis (RA) wordt gekenmerkt door pijnlijke en ontstoken gewrichten. De behandeling van 'reuma' wordt bemoeilijkt door dat de oorzaak van de gewrichtsontsteking nog onbekend is. De onstekingsreactie kan gericht zijn tegen lichaamsvreemde stoffen (antigenen) afkomstig van virussen, bacteriën, of uit ons voedsel of door een dwaling van het afweersysteem (b.v. kruisreactie), tegen de bouwstenen van het kraakbeen; de proteoglycanen en collagenen. In RA en in sommige experimentele artritis modellen zijn aanwijzingen hiervoor gevonden echter blijft de vraag of zij de oorzaak dan wel het gevolg zijn van de ontsteking? Vooralsnog is de argumentatie niet sluitend welk proefdier nu model staat voor humane RA. Het zal duidelijk zijn dat een observatie in meerdere experimentele artritis modellen gezien, aan betekenis wint.

Globaal zijn drie niveaus te onderscheiden in het ontstekingsproces. Ten eerste, de instroom van ontstekingcellen in het gewricht. Hierbij zijn adhesie molekulen en chemokines van belang. Ten tweede, de cytokinen voor de communicatie tussen alle betrokken cellen in het ontstoken gewricht en daar buiten. Ten derde, de enzymen welke verantwoordelijk zijn voor kraakbeenmatrix destructie. Het zal duidelijk zijn dat deze drie niveaus nauw verbonden zijn met elkaar. In de strijd tegen RA staan we voor de keuze om het ontstekingsproces bij te sturen dan wel te stoppen of de kraakbeendestructie te verminderen en herstel te bevorderen.

In mijn studie staat het cytokine, interleukine-1, centraal. Interleukine-1 (IL-1) bestaant uit twee sterk homologe 17 KDa grote eiwitten die voor een zelfde membraan-receptor competeren. Gebaseerd op *in vitro* waren er al aanwijzingen dat IL-1 mogelijk een belangrijke rol speelt in de kraakbeenschade en het ontstekingsproces.

In **hoofdstuk 2** toonden wij aan dat incubatie van gewrichts-kraakbeen van de muis met recombinant muize IL-1α of IL-1ß leid tot proteoglycan verlies. Dit verlies was chondrocyt (kraakbeencel) gemedieerd en werd veroorzaakt door een verhoogde proteoglycan afbraak en een sterk verminderde proteoglycan synthese. Deze verschijnselen traden ook op na injectie van het interleukin-1 in het muize gewricht (**hoofdstuk 3**). Een eenmalige IL-1 injectie in de knieholte remt de proteoglycan synthese in het kraakbeen sterk gedurende 2 á 3 dagen. Tevens vind er een verhoogde afbraak plaats de eerste dag na de injectie. De milde ontsteking die volgde op IL-1 was onvoldoende ernstig om verantwoordelijk te zijn voor de schade. Herhaalde IL-1 injecties veroorzaakt een histologisch duidelijk waarneembare kraakbeenschade in het hele kniegewricht.

In **hoofdstuk 4** toonden we aan dat de verhoogde proteoglycan afbraak samenging met een verhoogde expressie van gelatinolytische enzymen in het gewrichtskapsel en kraakbeen. Tot op heden is het verantwoordelijke enzym voor de kraakbeen destructie echter nog niet geïdentificeerd. De door IL-1 geinduceerde kraakbeenschade was reversibel en herstel werd gekenmerkt door een sterk verhoogde proteoglycan synthese en een verminderde afbraak van de nieuw geproduceerde proteoglycanen.

Tijdens de eerste dag van de antigeen-geinduceerde artritis (AIA) in de muis vind er IL-1 productie plaats in het knie gewricht. Het behandelen van muizen met neutraliserende konijne antilichamen gericgt tegen muize IL-1 had geen effect op de initiele gewrichtszwelling en instroom van ontstekingscellen. De synthese van proteoglycanen werd echter volledig behouden door deze behandeling (**hoofdstuk 5**). Ook al ging de verhoogde kraakbeen afbraak onverminderd door de schade aan het kraakbeen was duidelijk verminderd door de behandeling. Het was van belang om op tijd te starten met de therapie. Werd de anti-IL-1 behandeling uitgesteld tot 24 uur na aanvang van arthritis dan kon de proteoglycan synthese niet meer beschermd worden (**hoofdstuk 6**).

Tumor necrosis factor (TNF) kan ook kraakbeenschade induceren alhoewel IL-1 veel potenter is in de muis. Interleukine-6 (IL-6) speelt mogelijk een rol als essentiele cofactor in de IL-1 geinduceerde proteoglycan synthese remming maar induceert zelf geen kraakbeenschade in de muis. In **hoofdstuk 7** laten we zien dat neutralisatie van TNF en IL-6 met antilichamen geen effect had op AIA en de niet-immunologisch gemedieerde zymosan-geinduceerde artritis (ZIA). Echter ook in ZIA was IL-1 verantwoordelijk voor de proteoglycan synthese remming. Het blokkeren van de IL-1 activiteit met een humaan IL-1 receptor antagonist protein leverde een zelfde resultaat op. Deze gegevens zijn recentelijk bevestigd in andere muizen modellen op ons laboratorium: de collageen-geinduceerde artritis (CIA) en de immuuncomplex-artritis (ICA).

De ziekteactiviteit in RA fluctueert in de tijd en het ziekteverloop kan gepaard gaan met opvlammingen die ernstige gevolgen kunnen hebben voor het gewricht.

Interleukin-1, lokaal of systemisch toegediend in de muis was in staat tot een vergelijkbare kortstondige opvlamming van AIA (**hoofdstuk 8**). IL-1 was ook in staat tot gewrichtszwelling en influx van ontstekingscellen in ZIA. Dit, en het feit dat IL-1 nog steeds opvlammingen induceerde van AIA na anti-lymfocyt behandeling maakte het aannemelijk dat de macrofagen de IL-1

geinduceerde opvlammingen medieerden. Dit werd bevestigd door IL-1 geinduceerde opvlammingen in gewrichten met een macrofaag rijk infiltraat (concanavalin-A behandeld).

De rol van IL-1 in de antigeen-specifieke opvlammingen van AIA werd nader onderzocht met neutraliserende antilichamen. Het blokkeren van IL-1 activiteiten leidde tot een duidelijk vermindering van de gewrichtszwelling en de kraakbeenschade **(hoofdstuk 9)**.

Gebaseerd op deze bovenstaande bevindingen in de muis acht ik het niet onwaarschijnlijk dat het blokkeren van IL-1 in het ontstoken gewricht een zeer belangrijke bijdrage kan leveren in de behandeling van rheumatoïde arthritis. Mogelijk is IL-1 de verantwoordelijke mediator (cytokine) voor de remming van de proteoglycan synthese in humaan RA kraakbeen. Een anti-IL-1 behandeling kan mogelijk leiden tot een verminderde schade aan het gewrichtskraakbeen en IL-1 speelt potentieel een nog grotere rol in het ontstekingsproces en de gerelateerde kraakbeenschade tijdens de veel voorkomende opvlammingen van RA.

DANKWOORD

Toen ik tijdens de bereiding van een stoofpotje de keuken rond keek trof mij de gelijkenis met mijn werk. Ook wij staan als een kok in potjes en pannetjes te roeren en creëren uit de verschillende ingrediënten onze gerechten. Welbeschouwd is de wetenschap nauw verwant aan de kookkunst en vice versa. Als in elke kunstvorm probeert de wetenschapper door combinatie en variatie, grenzen te verleggen. Het is mij opgevallen dat in beide disciplines het verslag veel overeenkomsten vertoont. De ingrediënten en het instrumentarium worden zorgvuldig beschreven en samen met een gedetailleerde receptuur moeten zij borg staan voor de met veel verve gepresenteerde resultaten. Ook in de kookkunst is de publikatie druk sterk toegenomen.

Eten en wetenschap gaan hand in hand en een (Indonesisch) diner is dan ook onontbeerlijk tijdens een congres. Hier geldt dat een heerlijk gerecht smaakt naar meer.

Dit proefschrift was dan ook nooit tot stand gekomen als niet alle ingrediënten aanwezig waren op de afdeling Reumatologie. Daarom wil ik dan ook IEDEREEN bedanken die mij terzijde stonden om van dit stoofpotje een meergangen menu te maken en mij voorzagen van een A-klasse kwaliteit.

Door zijn aanwijzingen (kneepjes van het vak) en de in mijn gestelde vertrouwen heeft Wim van den Berg als chef de cuisine mij met woord en daad bijgestaan. Met Leo van de Putte als keurmeester op de achtergrond waakte ik ervoor niet al te grote misbaksels en rookwolken te produceren.

Als iedere kok had ik ook mijn maatje, Onno Arntz. Zonder zijn inzet, zelfwerkzaamheid en kritisch volgen waren de gerechten zeker niet gaar geworden, daarnaast voorkwam hij regelmatig dat menig potje zou aanbrandden. Al gauw werd FONNO een vertrouwd huismerk.

Velen onder jullie hebben smaak toegevoegd aan mijn gerecht. Met name Peter van Lent had creatieve ideeën en strooide royaal met peper en zout. Peter van der Kraan en Leo Joosten keken regelmatig onder de deksel en kruidde de gerechten naar believen. Het toetje dat Andrew als leerling kok heeft gemaakt past prima bij het menu.

Het gezegde 'verandering van spijs doet eten' was ook in mijn onderzoek van toepassing. Ivan Otterness stelde mij in staat om in de Amerikaanse keuken van Pfizer te kijken hetgeen geen fastfood restaurant bleek te zijn. Hij stak veel tijd en energie in mijn kookkunsten, maakte mij deelgenoot van zijn creatieve potjes en stimuleerde mij voor in lengte van dagen. Marcia Bliven wil ik bedanken omdat wij altijd op haar konden rekenen. Alhoewel zij een kleine eter is was het altijd prettig om met haar uit te gaan.

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Tot besluit wil ik opmerken dat een maaltijd pas tot zijn recht komt in gezelschap van vrienden. Vandaar dat ik mijn (w)etenschappelijke proeve van bekwaamheid wil besluiten met

EET SMAKELIJK!

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HUN PASSIE WERD ONZE MISSIE

De passie van bovenstaande legendarische wetenschappers om manieren of middelen te vinden, waarmee zieke mensen konden worden geholpen, is voor ons een missie geworden.

Het doel van Pfizer is haar continuïteit als researchgerichte organisatie te waarborgen, door optimaal ondersteuning te bieden aan de medische professie om zieken te genezen, lijden te verzachten of anderszins de levensomstandigheden en het welzijn van patiënten te bevorderen.

Dit streven verplicht ons tot het ontwikkelen van geneesmiddelen en therapieën die kwaliteit, betrouwbaarheid en gemak in zich bergen.

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